METHIONINE METABOLISM IN FILARIAL WORMS

BY

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Date: September, 1980

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Thesis submitted to the Department of
Biochemistry, University of Ghana,
Legon in fulfillment of
the requirements for the
degree of Doctor
of Philosophy.

The experimental work for this thesis was conducted at the University of Vermont, U.S.A., under the direct supervision of Dr. J.J. Jaffe of the Department of Pharmacology.

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LIST OF ABBREVIATIONS

AdoHcy, AdoMet	S-Adenosylhomocysteine, and - methionine
ADP, ATP	Adenosine 5'-di-, and triphosphate
BHC	Gamma-benzenehexachloride
B12	Cyanocobalamin
BSS	Balanced salts
C	Carbon
^{o}C	Degree Centrigrade
Ca	Calcium
Cd	Cadmium
Co	Cobalt
CoA	Coenzyme A
CO_2	Carbon dioxide
CH_2	Methylene
CH_3	Methyl
Conc.	Concentration
cpm	Counts per minute
D	Dextrorotatory
DL	Racemic mixture
DDT	Dichlorodiphenyltrichloroethane
DEC	Diethylcarbamazine
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
d.p.m.	Disintegration per minute
DTT	Dithiothreitol
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid

FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
g	Earth's gravitational field, 980.6 cm/sec^2
GSH	Reduced glutathione
h	Hour
HCl	Hydrochloric acid
KCl	Potassium chloride
KCN	Potassium cyanide
Km	Michaelis constant
L	Levorotatory
M	Molar
mCi	Milli curie
MCP	Methyl-accepting Chemotaxis Protein
Mel W	Pentylthiarsaphenylmelamine
MEME	Minimum essential medium, Eagle
Met	Methionine
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mmol	Millimole
Mn	Manganese
mRNA	Messenger RNA
MSbB	4-Melaminyl-1- (methylolcylo (ethylenedithiastibina) benzene
MTA	5'-Methylthioadenosine
N	Nitrogen

NaCl	Sodium chloride
NAD^+,NADH	Nicotinamide adenine dinucleotide and its reduced form
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nCi	Nano curie
NH_4OH	Ammonium chloride
Ni	Nickel
PDE,PME	Phosphatidyl-monoethyl-, and -dimethyl-ethanolamine
pH	- Log H^+ concentration
P(i)	(Inorganic) phosphate
PPO	2,5-Diphenyloxazole
Rb	Rubidium
RNA	Ribonucleic acid
RNase	Ribonuclease
S	Sulphur
sec	Second
SO_4^{2-}	Sulphate
SSC	NaCl and sodium citrate
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle
THF	Tetrahydrofolate
TKM	Tris, KCl and $MgCl_2$
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
TW Sb	Antimony a, a-dimercaptosuccinate
UTP	Uridine triphosphate

UV	Ultraviolet
v	Volume
Vmax	Maximum velocity
w	Weight
Zn	Zinc
ul	Microlitre
um	Micrometre
%	Percent

ACKNOWLEDGEMENTS

My thanks are primarily due to my external supervisor Dr. J.J. Jaffe of the Department of Pharmacology, University of Vermont, for his helpful advice and encouragement throughout this research. I should also like to thank him and his wife for making my stay in Burlington enjoyable.

The greatest indebtedness that I owe is to the World Health Organization for the financial support without which this project could not have been undertaken.

My indebtedness also goes to Prof. D.A. Bekoe, the Vice-Chancellor, University of Ghana, Legon and Prof. G.S. Asante, the Head of Department of Biochemistry, Legon for going through all the administrative tedium of setting me on a career course. I am also grateful to my supervisory committee members in the Department of Biochemistry, University of Ghana, Legon: Drs. K.K. Oduro (Internal Supervisor), F.N. Gyang and S. Asante-Poku for their valuable suggestions.

I should further like to express my appreciation and thanks to Dr. J.C.W. Comley, Mrs. L.R. Chrin and Mrs. R. Smith for their immense contribution to this work. Also deserving gratitude are Drs. Alan Eastman, Lazarus Durkwa, Charles des Bordes, and Tshimanga N'Zagi, Mr. Donald Taylor, the entire faculty members of the Department of Pharmacology especially Drs. J.J. McCormack and R. Newman, the staff of the International Student Office, University of Vermont, and Mr. Gabriel B. Fosu of Brown University, Providence, Rhode Island. Finally, but not the least, much thanks go to Miss Gail Fairbanks and Mr. Kofi Gyau for typing the manuscript.

ABSTRACT

Adult filariae apparently lack the vitamin B12-dependent and -independent methyltransferases for the de novo synthesis of methionine and seem to meet their requirement of this amino acid from an exogenous source and the activity of S-adenosylmethionine(AdoMet):homocysteine S-methyltransferase. The properties of filarial AdoMet:homocysteine methyltransferase were similar to the analogous microbial enzyme. However, adult filariae possess the enzymatic capability to metabolize methionine to cyst(e)ine.

When incubated in the presence of L-[CH_3-^{14}C]methionine to induce them to synthesise AdoMet, adult Dirofilaria immitis incorporated the radiolabel into phospholipid and protein fractions.

The significance of filarial methionine metabolism especially with regard to its suitability as a potential target for antifilarial chemotherapy is discussed.

Chapter 1

LITERATURE REVIEW AND INTRODUCTION

A. Life Cycle of Filariae

Filariae are parasitic nematodes of the Order Filariidea which cause filariasis in millions of people in tropical countries (1). Although filariasis usually refers to infections with Wuchereria bancrofti, Brugia malayi and Brugia timori, in the present context it is used more broadly to include those of Onchocerca volvulus, Loa loa, Mansonella ozzardi, Dipetalonema perstans and Dipetalonema streptocerca.

Filarial worms pass through five developmental stages in their life cycle. The adult or fifth-stage worms take months to mature, are long-lived and do not undergo asexual multiplication in the definitive host. After mating the viviparous females discharge, according to the species, numerous sheathed or unsheathed microfilariae or first-stage larvae. Except for the microfilariae of O.volvulus and D.streptocerca which invade the skin, the others circulate in the peripheral blood, and they may exhibit different kinds of circadian periodicity (2). When ingested by the appropriate insect vector with its blood meal, the microfilariae of all species migrate either to the flight muscles, the Malpighian tubules, or the abdominal fat bodies, where they metamorphose intracellularly, without multiplying, into the infective filariform (third-stage) larvae. Vectors of W.bancrofti belong to the genera Culex, Aedes and Anopheles while Mansonia mosquitoes are the main secondary hosts of Brugia. Various species of Simulium, Chrysops and Culicoides are the respective intermediate hosts of Onchocerca, Loa and Dipetalonema. The

infective larvae migrate to the salivary gland in the head of the insect vector, and enter the definitive host through the proboscis when the insect takes its blood meal. Once in the final host the larvae migrate to their preferred sites: skin (Onchocerca, Loa, D.streptocerca), lymphatics (Wuchereria, Brugia), or body cavity (Mansonella, D.perstans) and there undergo two moults before they become mature parasites.

B. Pathogenesis of Filariasis

Characteristic features of onchocerciasis include nodulization, onchodermatitis, hanging groin, hernia, elephantiasis and eye lesions (3). The severity of the disease is a result more of reaction to the presence of microfilariae than to the adults. The inception of onchocerciasis might go unnoticed because the adults which live in the subcutaneous tissues may be unpalpable (4). As the disease progresses distinctive nodules or "onchocercomas" appear in which are found dead or dying worms (5). Onchodermatitis, which in some patients may be asymptomatic, is prominent at this stage. Characteristically, there are pruritis, papulosis, dermal thickening and eventually pachydermia which gives a senescent and emanciated look to the patient. Chronic skin lesions cause depigmentation called leopard skin especially over the legs.

Pachydermia predisposes usually adult males to hernia, pseudo-adenolymphocele or "hanging groin". The latter is a pendulous sac containing inguinal or femoral lymph gland which is known to cause scrotal elephantiasis in some endemic areas of Africa.

Ocular onchocerciasis is the most devastating aspect of this form of filariasis; it is an inflammatory reaction in the eye tissues to dead microfilariae. Conjunctivitis, corneal sclerosing keratitis

and anterior uveitis are common features. Invasion of the posterior chamber of the eye may lead to the atrophy of the optic nerve and the retina (3,6,7), resulting in "river blindness".

Although infection with Wuchereria and Brugia may be asymptomatic in some individuals, the acute phase is marked by lymphangitis, fever, lymphadenitis, lymphoedema and elephantiasis of limbs, genitalia and breast. Cryptic infection with microfilariae in especially the lungs and hyperplastic lymph nodes is a common aetiology of tropical eosinophilia (8-13).

"Calabar swelling", subcutaneous and transocular migration of adult worms and hypereosinophilia are the main symptoms of loaiasis. Occasionally there might be albuminuria which may be aggravated by diethylcarbamazine treatment. Invasion by microfilariae of the cerebrospinal fluid may predispose persons with high microfilaremia to meningo-encephalitis (14). Recent evidence (7) has associated the once considered non-pathogenic dipetalonemiasis and mansonelliasis with eosinophilia, papulosis and pruritis.

C. Socio-Economic Aspects of Filariasis

The pathogenesis of the different forms of filariasis reveals that the disease is capable of producing severe debility, the socio-economic consequences of which must be enormous.

The economy of countries in the filariasis-endemic regions of the world is primarily agricultural, relying heavily upon manual labour, malnutrition and infectious diseases, among other factors, reduce the quality of the labour force resulting in a lower output.

In many parts of West and equatorial Africa, more than 50% of the inhabitants are infected with onchocerciasis; 30% have amblyopia and 4-10% are blind (15). In some villages of Upper Volta and Northern Ghana the prevalence of blindness in adult males may reach 30-40%, whereas the rate of blindness in onchocerciasis-free zones is between 0.5-1.0%. The average proportion of blind persons in endemic areas is 10%, as against 0.2% in regions free of onchocerciasis (16).

Morbidity due to onchocerciasis has led to depopulation of fertile land near rivers where Simulium damnosum breeds. It is estimated that 65,000 km^2 of the Volta River basin with a potential annual agricultural output of about US $30 million are deserted (17). Tourism which provides some developing countries with substantial foreign exhange is also seriously affected because the tourist attractiveness of endemic areas is considerably reduced.

Filariasis, like other tropical diseases, is therefore partly responsible for impeding progress and economic development in many developing countries. Its eradication or control would have a tremendous socio-economic impact.

D. Treatment of Filariasis

Chemotherapy and chemoprophylaxis play an important role in the control of some parasitic infections. Although a vaccine for malaria perhaps is in sight (18), for the majority of the parasitic diseases a practical method of immunization against them may only be realized in the distant future. Until then, drugs will continue to play a significant role in the control and treatment of these infections.

Diethylcarbamazine(1-diethylcarbamyl-4-methylpiperazine) and suramin (trisodium salt of 8,8'-(3'',3'''-ureylenebis(3''''-benzamido-4''''-methylbenzamido))-bis-1,3,5,-naphthalenetrisulphonic acid) are the only effective drugs presently available for the treatment of human filariasis, and knowledge about them, including their clinical pharmacology, were recently thoroughly reviewed by Hawking (19,20).

(a) Diethylcarbamazine: The antifilarial activity of diethylcarbamazine (DEC) was discovered by Hewitt and colleagues (21) in 1947 using a cotton rat, Sigmodon hispidus, naturally infected with Litomosoides carinii. It was found that neither DEC nor sera derived from treated animals exerted any antifilarial action in vitro. By contrast a profound and rapid effect was obtained when DEC was administered intravenously to L.carinii-infected cotton rats; there was an 80% reduction in microfilaremia within two minutes (22,23). DEC is similarly potent in vivo against the microfilariae of W.bancrofti, B.malayi, O.volvulus, D. streptocerca, L.loa and Dirofilaria immitis (2,3,6,9,14,23-25).

It was established by a number of investigators that DEC had no activity against adult filariae in vitro. The susceptibility of adult worms to DEC in vivo was found to be more variable than that of the microfilariae and appeared to be species-dependent. Hewitt et al. (21) reported that DEC was a macrofilaricidal agent against L.carinii, but this finding could not be confirmed by Hawking et al. (22). The latter investigators suggested the duration of infection as the cause of the anomaly; since Hewitt's group used rats with comparatively old infections and death of the worms might have been due to senility or to a host

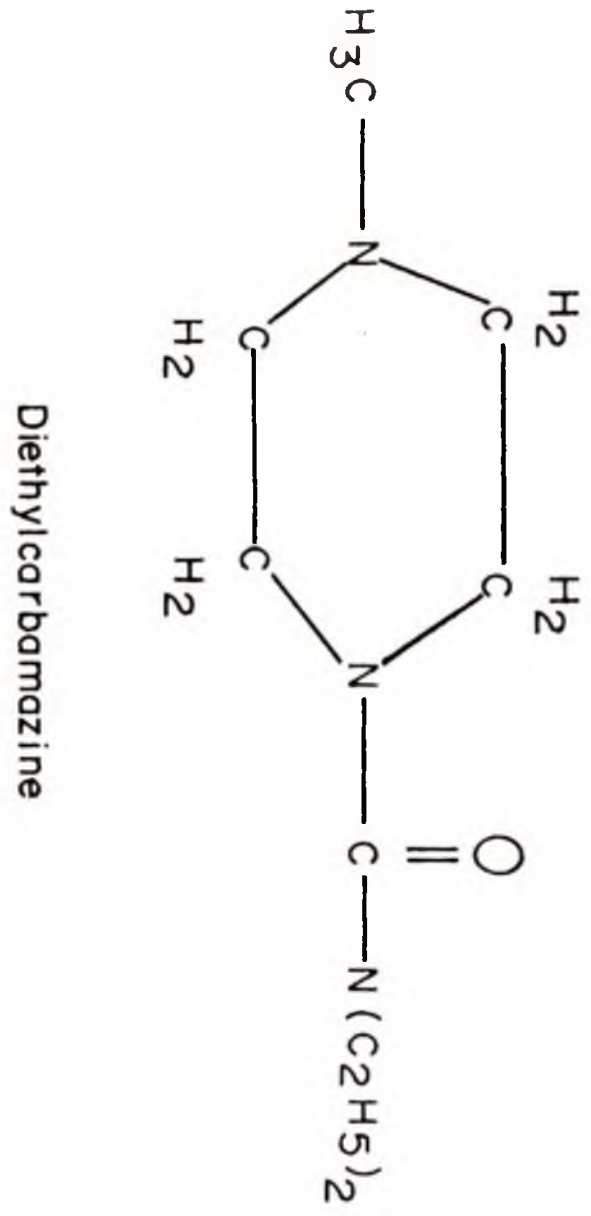
immune response. Treatment of patients infected with O.volvulus using ordinary dose regimes of DEC showed no adverse consequences on the adult worms (26); however, some of the adult filariae were destroyed but only after prolonged intensive treatment (21,27). It was reported that adult L.Loa, D.perstans and W.bancrofti could be killed by DEC as evidenced by the prolonged absence of detectable macrofilaremia after administration of the drug (23,28-30). Adult B.malayi and D.streptocerca are also susceptible to DEC, although a high dosage is required to kill the latter (8,10,14, 31-33).

Absorption & Distribution: Although DEC is normally given orally, it can be applied topically over nodules in onchocercal cases (20). Whatever the route of administration the drug is rapidly absorbed. According to estimation of Rees et al.(34) after a single oral dose of 200-400mg to man, a peak plasma level was reached within two hours; after a dose of 200 mg the plasma half life was 8.1 $\pm$ 3.5 hr and 11.7 $\pm$ 2.3 hr after 800 mg of DEC. Although a maintenance level could be attained with large daily doses, there was no tendency for the drug to accumulate. DEC is apparently widely distributed, with liver, kidneys, adrenal glands, muscle, gastrointestinal tract, salivary glands and brain exhibiting high levels (20).

Metabolism & Excretion: Using ^{14}C-labelled DEC, Bangham (35) showed that the piperazine ring was entirely eliminated within 24-30 hr in the urine. Analysis of urinary metabolites by paper chromatography and ion-exchange column chromatography following intravenous administration of DEC showed that 10-20% of the compound was excreted intact, 8-15% as diethylcarbamylpiperazine, 2-5% as methylpiperazine and 1.6% as piperazine

Figure 1

The Chemical Structure of Diethylcarbamazine



Diethylcarbamazine

and the remaining 60% as unidentified compounds. Subsequent investigations by Faulker and Smith (20) revealed the identity of the unknown metabolites as diethylcarbamazine-N-oxide, 50%, and 1-ethylcarbamyl-4-methylpiperazine, 23%. Although metabolism is rapid and extensive, no metabolite so far isolated appears to have a sufficiently profound effect to account for the extraordinary speed of action on the circulating microfilariae.

Mode of Action: The exact mode of action of DEC is uncertain. Current evidence suggests that it may be exerting an opsonizing effect either by causing exsheathment or by damaging the cuticle and thereby unmasking antigenic determinants of microfilariae and susceptible adult worms leading to phagocytosis by the host's immune system (23,27,36). Histological studies on cotton rats infected with L.carinii have revealed that the action of DEC in vivo is associated with aggregation of the microfilariae in the liver where they are trapped by phagocytes leading to their eventual dissolution (23,37,38). Implicit in this opsonin-like action might be an immunological response since it has been observed that microfilariae in serous cavities were apparently not destroyed by DEC (24). Current work in experimental filariasis suggests that immunity may be a prerequisite for the expression of the microfilaricidal activity of DEC. Kobayashi et al. (37) found that the drug did not affect microfilariae of L.carinii injected into normal cotton rats or circulating larvae emerging from adult worms transplanted into naive hosts. In the latter case, however, microfilariae were cleared from the circulating blood fifteen days after transplantation of mature worms. Treatment of cotton rats with immuno-suppressive agents followed by DEC, however,

gave inconclusive results. DEC administered simultaneously with anti-lymphocyte serum to infected cotton rats was also ineffective. DEC treatment was efficacious only after termination of antilymphocyte serum treatment.

Because DEC is a piperazine derivative, it has been postulated that this drug may possess antihistaminic properties as was demonstrated in a series of related compounds (39-42). On the other hand, experiments with rats and calves suggest that DEC rather than being antihistaminic may be inducing the release of vasoactive mediators, including histamine (43).

An action of DEC on the neuromuscular system is also possible particularly as acetylcholine and cholinesterase activity have been detected in filariae (44). Piperazines have been shown to hyperpolarize isolated muscle cells of Ascaris lumbricoides var. suum with consequent flaccid paralysis, the effect of which can be reversed by adding acetylcholine (45-49).

Toxicity: DEC is not without side-effects. Two types of toxic reactions are noted in DEC therapy, those related to the direct effects of the drug and those secondary to the death of the microfilariae and/or macro-filariae.

In uninfected persons, DEC can cause gastrointestinal disturbances especially if administered before meals. Other documented adverse reactions include headache and drowsiness.

In patients infected with filariae the side-effects are variable with the severity depending upon the form of filariasis and the worm burden. Side effects are particularly prominent in onchocerciasis (23)

and include swelling, oedema, pruritis, hyperpyrexia and inflammatory reactions, the latter complicating the treatment of ocular onchocerciasis (50,51).

(b) Suramin: In 1920 Bayer introduced suramin for the treatment of human trypanosomiases. Its filaricidal efficacy particularly against O.volvulus was discovered in 1945 by Van Hoof and colleagues in Zaire (then Congo) during field trials (23).

Contrary to the findings of Hawking (23), those of Lammler et al.(52) indicated that suramin could kill L.carinii in Mastomys natalensis within six weeks. The two weeks observation period other workers had utilised was too short a duration for any effect to be discernible.

Infective and other immature stages of L.carinii are very sensitive to suramin (19). Subcutaneous injection of 40 mg/kg/day suramin for five days starting one, two, or four weeks after infection completely removed all traces of infection. Lower doses given for five days immediately after infection did not kill all the worms.

There was also a delayed response by adult O.volvulus and W.bancrofti to suramin. Female worms were more susceptible than the males, probably due to their more active metabolism (53). The action of suramin on the other filarial parasites of man is unknown.

Direct microfilaricidal activity of suramin is unusual, although microfilariae of O.volvulus are eventually cleared from the body possibly by living out their normal life span (10-18 months), the macrofilaricidal effect obviating replenishment. The development of infective larvae of O.volvulus in chimpanzees is likewise terminated by suramin (19). In

two human volunteers, however, no chemoprophylaxis could be demonstrated probably due to the short observation period (54).

With the possible exception of Simulium damnosum (the vector species for O.volvulus) no effect of suramin on the development of microfilariae in the other arthropod hosts, mosquitoes, tabanids and midges, has been reported. In the case of Simulium the apparent microfilaricidal effect is thought to have been exerted via the suramin-treated patients on whose blood the vectors fed (55).

Absorption & Distribution: The limited intestinal absorption of suramin when given by mouth and the acute local inflammatory reaction following subcutaneous or intramuscular injection has made the intravenous route the practical course of administration.

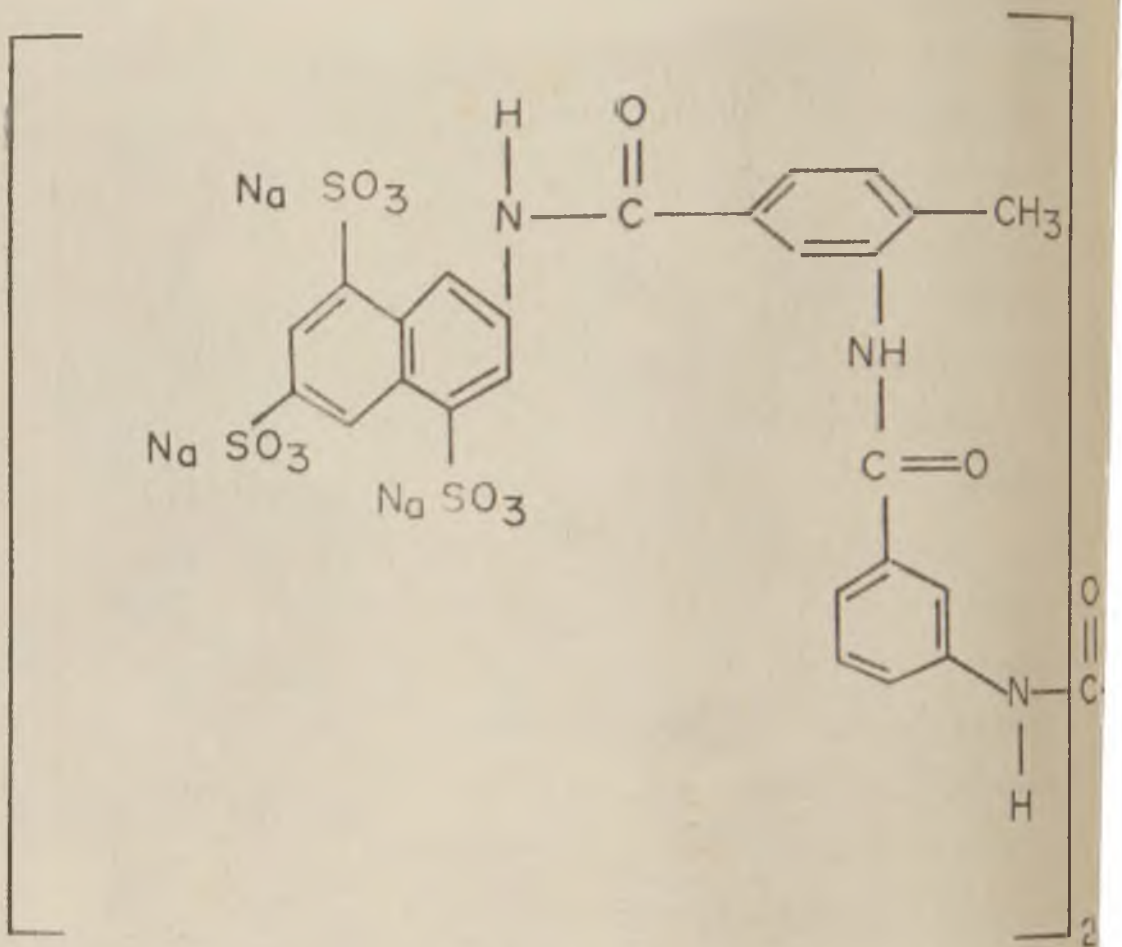
After intravenous injection suramin combines rapidly with serum proteins, and most of it circulates in the blood in this bound form. Some of it, probably combined with protein, is taken up by the cells of the reticuloendothelial system. Its presence in the epithelium of the proximal convoluted tubules of the kidney has also been demonstrated (19).

Metabolism & Excretion: Although excreted by the kidneys, most of an administered dose persists in the body for a prolonged period, due to its extensive binding to plasma proteins. Apparently suramin does not undergo any form of metabolic transformation in the body, suggesting that its chemotherapeutic activity is due to the intact molecule.

Mode of Action: The slow onset of suramin's macrofilaricidal activity, with a latency of 5-6 wk, parallels its delayed trypanocidal action

Figure 2

The Chemical Structure of Suramin



Suramin

where it is effective only after six cell divisions. In trypanosomes, it is believed that suramin primarily inhibits DNA and RNA polymerases probably by bridging the active sites (19). The presence of multinucleated trypanosomes among those exposed to suramin also suggests that cytoplasmic cleavage is inhibited. The enzymatic mechanism responsible for this subdivision is thought to be sensitive to suramin as has been demonstrated in the bacterium Streptococcus foecalis (56).

Sensitivity of these important processes to suramin might explain its antifilarial action. Since it is only the reproductive cells which are actively multiplying in the macrofilariae, it is likely that they would be the sites of suramin's action. In addition, the inhibitory action of suramin on some important key metabolic enzymes may further serve to augment its primary effect. Suramin,was found to inhibit hexokinase (19), glycerophosphate dehydrogenase and glycerophosphate oxidase (252). Jaffe and colleagues (57) have found that dihydrofolate reductases from four species of adult filarial worms, including O.volvulus, were sensitive to inhibition by suramin in the 2-10 uM range. Suramin was also found to be a potent inhibitor of two other folate-related enzymes from adult Brugia, 10-formyltetrahydrofolate dehydrogenase and 5-formyltetrahydrofolate cyclodehydrase (58).

Toxicity: This has been studied in depth in patients with trypanosomiasis. The immediate reaction to an intravenous injection of suramin characterized by nausea, vomiting, sweating and/or coma, is followed by a reaction with the following features: hyperpyrexia, photophobia, transient conjunctivitis, gastrointestinal problems and nephrotoxicity (19,23). Albuminuria, exfoliative dermatitis, prostration, chronic diarrhoea and

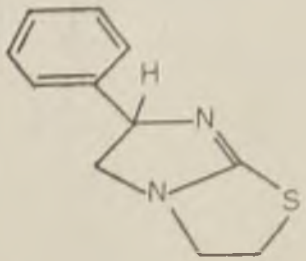
inflammatory reactions are some of the severe side effects associated with suramin therapy. The allergic reaction is triggered by the sensitization of the host to the dead filarial worms, often predisposing some ocular onchocerciasis patients to optic atrophy (51).

DEC is the drug of choice for mass chemotherapy. However, its side effects are a stumbling block to mass treatment (14). New antifilarial drugs with higher efficacy and fewer side effects are urgently needed. Meanwhile, a wide variety of presently available parasitic drugs have been tested or are being reevaluated as antifilarial agents.

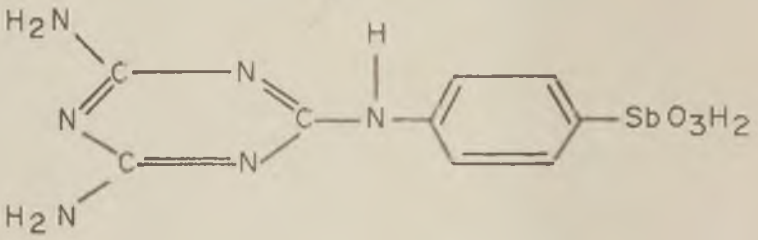
(c) Antimony Compounds: In addition to their use in the treatment of trypanosomiasis and schistosomiasis, antimony-containing compounds were shown to be effective against D.immitis in dogs and W.bancrofti in man. Neostibosan was found among various antimony derivatives to be potent against L.carinii in cotton rats and human W.bancrofti. It was also effective against L.loa but not against O.volvulus (59,60). Friedheim (61) also showed that MSbB (4-melaminyl-1-[methylolcylo(ethylenedithiastibina)]-benzene) was effective against human W.bancrofti. Extensive field trials by Duke (62) on patients in West Africa with O.volvulus revealed the efficacy of TWSb (Antimony a,a-dimercaptosuccinate) and MSbB in the treatment of onchocerciasis. However, this promising lead could not be exploited owing to the low margins of safety of these antimony-based compounds.

Figure 3

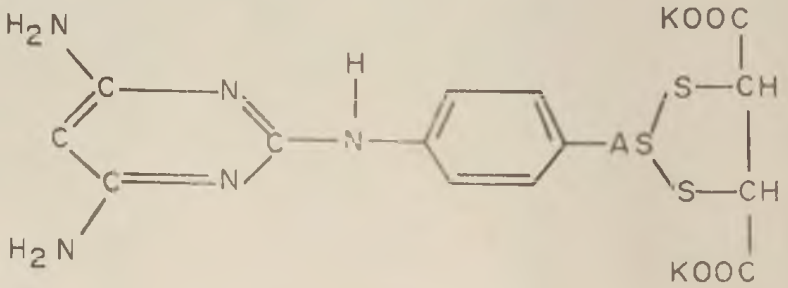
The Chemical Structures of Levamisole, 4-Melaminyl-1-(methylolcyclo(ethylenedithiastibina) benzene, and Pentylthiarsaphenylmelamine.



Levamisole



4-Melaminyl-1-(methylolcyclo(ethylenedithiastibina) benzene



Pentylthiarsaphenylmelamine

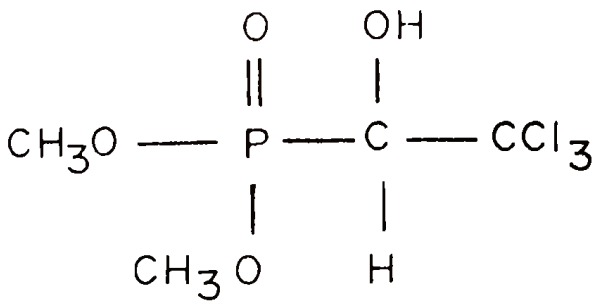
(d) Arsenic Compounds: Arsenicals also are efficient filaricides. Mel W (Pentylthiarsaphenylmelamine) introduced by Friedheim (63,64) was found to be macrofilaricidal against W.bancrofti and O.volvulus in man, but its sometimes fatal side-effects, however, precluded its further application. Thiacetarsamide killed macro- and microfilariae in patients infected with W.bancrofti. It was lethal to microfilariae of W.bancrofti and O.volvulus in vitro and is effective against adult D.immitis in dogs. Like Mel W, its clinical application was precluded because of its toxicity.

(e) Metrifonate: Dimethyl-(2,2,2-trichloro-1-hydroxyethyl)-phosphonate, originally developed as an insecticide, can kill blood fluke trematodes, particularly Schistosoma haematobium, as well as other helminths. It is a potent anticholinesterase in helminths as well as in insects. Metrifonate is a powerful microfilaricide against L.carinii in Mastomys natalensis (52,65). It is macrofilaricidal against Dipetalonema witeae in M.natalensis (37), but against D.witeae infection in the gird, Meriones persicus, metrifonate has no therapeutic efficacy (37). Denham and associates showed that the drug was macrofilaricidal against Brugia pahangi in cats (66).

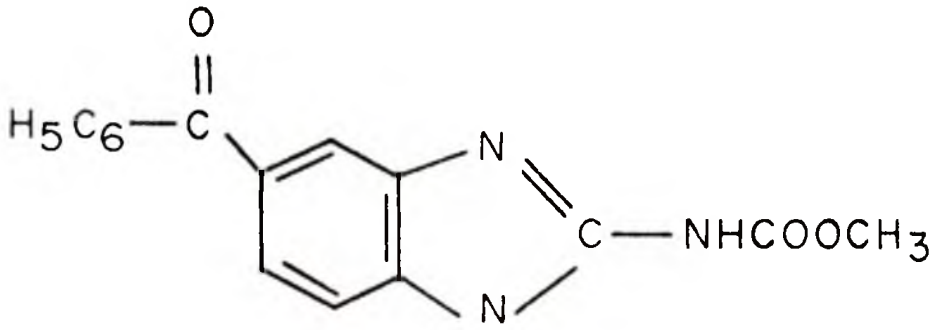
Its anti-onchocercal action was first reported by Salazar-Mallen (67) who found that a dose of 10 mg/kg daily for 6 days reduced the microfilaraemia of O.volvulus. In experiments with chimpanzees infected with O.volvulus, Duke (68-70) confirmed its microfilaricidal activity and also showed that a sustained high dose of 22 mg/kg daily for 6 days had no effect on the adult worms, despite noticeable host toxicity.

Figure 4

The Chemical Structures of Metrifonate and Mebendazole



Metrifonate



Mebendazole

(f) Mebendazole: Mebendazole [5 (6)-benzoyl-2-benzimidazole carbamate] is a broad-spectrum anthelmintic which interferes with the microtubular apparatus (71). The supposition is that the disruption of the microtubules leads to a breakdown of the transport and secretory mechanisms within nematode intestinal cells which in turn leads to an impairment of digestion and absorption of nutrients, thereby affecting the synthesis, uptake, transport and accumulation of basic substances. This mode of action can account for the earlier reported inhibition of glucose uptake by mebendazole (72).

Mebendazole was shown to be active against microfilariae and adults of L.carinii and D.witeae in jirds but was without effect on B.pahangi in jirds or O.volvulus in chimpanzees (73,74). Maertens and Wery (75) also found the drug ineffective against O.volvulus in humans when given as an oral dose of 100 mg twice daily for 6 days. Microfilariae of D.perstans in humans were susceptible to mebendazole after a two-week treatment.

(g) Levamisole: Levamisole is the levo-rotating isomer of 2,3,5,6-tetra-6-phenylimidazo(2,1-b)thiazole. It is a broad-spectrum anthelmintic agent which most probably acts by stimulating ganglionic structures in nematodes, resulting in a depolarizing type of neuromuscular blockage (76,77). It is also a powerful inhibitor of fumarate reductase in vitro (71). In addition levamisole is an immuno-stimulant (78-80), although its antiparasitic properties apparently do not depend on this action (81).

Lammler and associates (65) found levamisole to be filaricidal against L.carinii in M.natalensis. It was also found to be effective

against B.malayi, B.pahangi, W.bancrofti and D.witeae (82,83). The therapeutic index of levamisole in the treatment of experimental onchocerciasis was relatively low; though filaricidal, a dose of 10mg/kg/day given intramuscularly for 15 days to a chimpanzee, was intolerable (74). In human trials, a single dose of 120 mg/week for three weeks had no antifilarial activity in ten patients infected with O.volvulus. Reduction in microfilaeremia was observed in patients with B.malayi during a course of levamisole treatment, although upon cessation of chemotherapy there was an upsurge in the density of microfilariae.

(h) Vector Control: Where treatment with drugs is not feasible, the control of filariasis must depend on vector control, using such methods as:

(i) Sanitation, Urban Environmental Improvement & Education: The ideal strategy in vector control is the modification of the habitat, thereby preventing breeding of the vectors. In particular, poor environmental sanitation results in the creation of ecological conditions favourable for rapid multiplication of the vector population (84,85). Increase in density of Culex pipiens fatigans in many urban centres of the tropical world is a reflection of sanitary deterioration. Where elaborate sanitation systems have been introduced, the mosquito population has declined with a considerable reduction in the incidence of filariasis (14).

Health education programmes aimed at raising the awareness of the inhabitants in these regions to the values of sanitation would be important. However, health education, while effective, is a slow process and requires such elaborate training of the affected people that it is often viewed with indifference. For the time being, therefore, reliance must still be placed for the most part on other effective approaches.

(ii) Chemical Control: The use of insecticides in parasite vector control has been extensively investigated, especially in connection with malarial eradication programmes. Because of its long-lasting effects, dichlorodiphenyltrichloroethane (DDT) has been widely used to kill adult insects. However, DDT is now ineffective in C.p.fatigans control (86), as repeated application has caused the emergence of resistant strains. Development of resistance to other chlorinated hydrocarbon insecticides such as δ-benzenehexachloride (BHC) and dieldrin has also been reported (86). Organophosphorus compounds (e.g., fenthion, chlorpyrifos and chlorfenvinphos) were found to be effective against these DDT-resistant strains, although resistance to the organophosphate compound malathion in C.p. fatigans from Douala, Cameroun, has now been reported (86).

In some instances, killing the larval insect stages may be preferential to adulticiding. For example, although the latter approach has usually been adopted for endophilic vectors, exophilic biters are better controlled with larvicides. Systematic application of 0.1 ppm DDT to streams in selected endemic areas of onchocerciasis in East Africa was potent in controlling Simulium (37,86). High-spreading oils e.g. Malariol HS (Shell Oil Co) has also been used. Mansonia larvae have been controlled by spraying Pistia stratiotes, the water lettuce, with the herbicide Simazine, since Mansonia larvae are in close association with this aquatic plant from which they derive oxygen. Like adulticides, resistance by larvae to some of these larvicides is known (86).

(iii) Biological Control: Because of the actual or potential harm to the environment by chemical agents used for vector control in addition to the development of resistance to some of these insecticides, the use of alternative methods has become imperative.

Effective agents for the biological control of filarial vectors include certain species of larvivorous fish such as Gambusia affinis, Lebistes reticulatus and Nothobranchius taenipygus (87,88). Other promising agents are: a strain of Bacillus thuringiensis; certain fungi, such as Coelomomyces stegomyiae, Lagenidium giganleum, Metarrhizium anisopliae and the mermithid nematode, Reesimermis nielseni.

The wide-spread application of biological agents for vector control, though, poses potential direct and indirect hazards to man (88).

(iv) Genetic Control: Research efforts directed towards the development of sterile-male techniques and other forms of genetic manipulation for the control of mosquito and other vectors of disease seem encouraging. Some experimental results are encouraging for including such methods in future control programmes (37,89,90).

E. Metabolism of Filarial Worms: Currently employed chemotherapeutic agents in filariasis leave much to be desired. Effective and less toxic filaricides are urgently needed. Like other helminth parasites, adult filariae do not undergo asexual multiplication within their mammalian hosts, but instead mate to produce offspring which undergo further development within intermediate hosts. Therefore it might be expected that if a new efficacious macrofilaricide were developed, not only would it be a much needed curative agent but as a consequence of its wide application the transmission cycle of filariae would be interrupted.

Such a drug could be found by empirical screening of all sorts of chemical compounds. Alternatively, basic knowledge of the intermediary metabolism of filarial parasites might reveal biochemical pathways which could be blocked selectively by appropriate drugs.

Except for the pathways of carbohydrate metabolism and energy production, the metabolism of filariae generally has not been a research priority. Carbohydrates apparently form the major energy reserve of parasitic helminths, including filariae. It is not surprising that compounds whose mechanism of action lies in the interference with carbohydrate metabolism, such as antimonials and arsenical, have played a significant role in the control of a number of helminthiases.

A striking feature of carbohydrate metabolism in all parasitic helminths is the partial breakdown of glucose, even under aerobic conditions. With reference to the filariae, except for L.carinii, all others studied so far i.e. B.pahangi, D.viteae, Dirofilaria uniformis, Chandlerella hawkingi, D.immitis and Setaria cervi, seem to be homolactate fermenters (83,91,92). L.carinii converted 80% of the carbohydrate utilized to lactic acid anaerobically, acetic acid production accounting for the remainder (93). The generation of these acidic substances had been alluded to previously by Taylor (94) and Earl (95); both investigators reported a drop in pH of the media in which adult L.carinii and D.immitis were maintained. Aerobically about one-half of the glucose consumed was converted to lactic acid the rest to acetic acid and possibly glycogen (83,93). Recently Rew and Saz (96) reported that microfilariae of L.carinii, D.viteae and B.pahangi also metabolized glucose aerobically to acetate and CO_2. In contrast to the adults all three species of

microfilariids have an aerobic requirement for motility but possibly not for survival.

The principal pathway of glucose metabolism in filariae is phosphorylative glycolysis down to phosphoenolpyruvate, the steps being similar to those in vertebrate tissues (97-99). Subsequently, in adults, relatively high pyruvate kinase activity favours the production of pyruvate associated with a net production of ATP. Pyruvate in turn is reduced to lactate in the presence of lactate dehydrogenase. Filarial phosphoenolpyruvate may also be metabolized to oxaloacetate by way of CO_2 fixation catalyzed by phosphoenolpyruvate carboxykinase (97,100). Apparently, however, the pyruvate kinase-mediated pathway seems to be favoured in most filariae (100). Although an oxidative pathway to produce NADPH and ribose is found in filariae (98,101) at present there is insufficient evidence to postulate a complete hexose monophosphate shunt. NADPH is usually linked with membrane stabilization (in which reduced glutathione plays a prominent role) and fatty acid synthesis. It seems unlikely that the latter metabolic role operates in filariae, since nematodes apparently rely on exogenous fatty acids.

Notwithstanding the detection of the tricarboxylic acid (TCA) cycle enzymes in adult filariae (102,103), it has been speculated that the low levels of aconitase and isocitrate dehydrogenase might relegate the TCA cycle to an insignificant position in the overall metabolism of adult filariae. Oya et al. (104) have intimated that the TCA cycle in helminth parasites might be concerned with transamination reactions; buttressing this notion is the detection in some helminths of glutamate dehydrogenase activity which generates α-ketoglutarate. On the other hand it has been reported in microfilariae that the TCA cycle functions, but at a very

low level of turnover (96). In addition to completely oxidizing glucose, microfilariae share with adult L.carinii the metabolic peculiarity of oxidatively decarboxylating pyruvate to acetate (83,96,103). This reaction might explain the oxygen dependence and generation of energy for microfilarial motility. It also might account for the aerobic dependency of adult L.carinii. As a consequence of fluoroacetate inhibition it has been suggested that a fraction of the aerobically produced CO_2 of this filaria comes from the complete oxidation of glucose (93). However, the activity of cytochrome **c** oxidase has not been detected in suspensions of L.carinii (83,93).

Fractionation of filarial lipids revealed that phospholipids are predominant, the major phospholipids being phosphatidylethanolamine, and phosphatidylcholine; phosphatidylinositol, cardiolipin, sphingomyelin and lysolecithin were minor components (105-107). Other classes of lipids found in filariae were di- and tri-acylglycerols, free cholesterol, cholesterol esters, fatty acids (106,108), ubiquinone(s) and short-chain isoprenoid alcohol(s) (109). One group of investigators reported that the composition of filarial fatty acids which was similar in all the species examined, differed significantly from that of the host (106). In contradiction, Warren and Daugherty (110) found that the lipid composition of parasitic helminths was variable and depended on the host species.

Phospholipid synthesis has not yet been shown explicitly in any filariid, even though it has been established in other helminth parasites (111). The recovery of radioactivity from the lipid fraction of D.immitis microfilariae incubated with [U-^{14}C]-glucose at least implies that filariae can synthesize phospholipids by way of glycerol (101).

Except for Ascaris lumbricoides which seems to possess the ability to manufacture fatty acids by way of the malonyl CoA pathway, all other helminths including filariae appear unable to synthesize fatty acids de novo, but instead are capable of lengthening preformed fatty acids by condensation with acetyl CoA (108,111).

Although it is generally believed that parasites lack the necessary pathways to synthesize sterol (111), some tryparosomes and filariae have been reported to synthesize cholesterol de novo (108,111). On the other hand, Comley et al. (109) were unable to detect radiolabelled squalene and cholesterol in extracts of adult B.pahangi and D.immitis that were incubated in the presence of [2-^{14}C]mevalonate. These latter investigators found that substantial radioactivity derived from [2-^{14}C]mevalonate was recovered in short-chain isoprenoid alcohol(s) which on TLC ran close to cholesterol; they suggested that the difference between them and Turner and Hutchison (108) with regard to conclusions about the cholesterol-synthesizing capacity of filariae might be due to the method used by the two groups to separate cholesterol. In contrast to their apparent inability to synthesize cholesterol, adult B.pahangi and D.immitis were able to synthesize radiolabelled ubiquinone 9 when they were incubated with [2-^{14}C]mevalonate. Ubiquinone 9 had earlier been identified in the lung worm Metastrongylus elongatus (112). The presence of ubiquinone and rhodoquinone have been reported in a number of helminth parasites (113). Although ubiquinone may substitute for menadione in D.immitis N^5,N^{10}-methylene-tetrahydrofolate (methyleneTHF) reductase assay (109), it is probable that its presence in filariae indicates a role similar to its participation in other eukaryotic and prokaryotic

mitochondrial electron transport. Interestingly, ultrastructural studies have shown that adult filariids have a large number of highly cristated, well developed mitochondria normally associated with tissues capable of oxidative phosphorylation (103). The possibility exists that these highly structured mitochondria in filariae may function in an electron transport-linked phosphorylation of ADP coupled to a pyruvate dehydrogenase complex.

Lipid oxidation has been demonstrated both in A.lumbricoides eggs and free-living stages of other parasitic nematodes (111). This is not surprising since these developmental stages have functional β-oxidation and TCA cycles. Even though Subrahmanyam (105) suggested that triglycerides may be important energy reservoirs in L.carinii, it is unlikely that the oxidation of the acyl moieties of glycerides would account for this as the complete β-oxidation and TCA-cycle enzymes apparently are absent in adult parasitic nematodes (111); degradation of glycerol via glycolysis seems more likely (114).

Turning to other areas of filarial metabolism, Jaffe and Doremus (101) found that D.immitis microfilariae readily incorporated adenine, adenosine, uridine, and uracil as well as orotic acid into RNA but not into DNA. Utilization of preformed thymidine was not detected. Identical findings were reported by Chen and Howells (115) who studied adult B.pahangi and by Simpson and Lawrence (116) who studied Brugia patei laevae developing in Aedes togoi. De novo synthesis of thymidylate seems probable as thymidylate synthase activity has been detected in filariae (117). It is also likely that filariae can synthesize uridylic acid from formation and decarboxylation of orotidylic acid (111). Although de novo synthesis of purine nucleotides by D.immitis microfilariae

could not be detected (101), more recent investigations by Jaffe and Chrin (117) have demonstrated that adult filariae have this capacity, using 10-formylTHF and 5,10-methenylTHF to donate, respectively, carbons 2 and 8 of the purine ring. Wong and Ko (118) earlier reported that the parasitic nematode Angiostrongylus cantonensis can synthesise purine ribonucleotide de novo. Adult B.pahangi and D.immitis apparently cannot synthesise folic acid de novo; instead, they appear to rely on a source of preformed folate derived from their hosts. These filariae can oxidise 5-methylTHF directly to 5,10-methyleneTHF which in turn is connected to other THF cofactors that participate in single-carbon donors as in the enzymatic synthesis of serine from glycine and thymidylate from deoxyuridylate in addition to the synthesis de novo of purine nucleotides (58). It is noteworthy that 5-methylTHF is the major form of folate present in the extracellular fluids that envelope and nourish filariae, at least in their vertebrate hosts (58). There is evidence that a severe deficiency state is harmful to adult filariae in vitro (58). Deprivation of vitamins A, E and B6 also has an adverse effect on filariae; for instance, vitamin B6 deficiency of albino rats caused a state of amicrofilaremia in L.carinii (119). On the other hand thiamine deficient hosts were more susceptible to infection with filariae (120).

As relatively little is known about filarial metabolism and physiology, a scientific working group on filariasis convened by the World Health Organization Special Programme for Research and Training in Tropical Diseases recommended supportive investigations into this area of the parasites. It was the belief of the panelists that studies of this nature could reveal hitherto unrecognized aspects of the filarial biology and chemistry that could lead to the synthesis of selective antifilarial

drugs. Accumulated experience in related fields strongly suggests that rational development of drugs is possible. It has been established in the area of anti-infective chemotherapy that clinically useful drugs exploit qualitative or quantitative differences between parasites and their hosts in some analogous metabolic or physiological process. The traditional approach to anti-infective drug development, however, has involved empirical screening (121) in which substances from a wide variety of sources are tested for general growth inhibitory activity; promising compounds are further tested to determine their chemotherapeutic potential.

With respect to the background for the specific biochemical studies of filariae that are the subject of this thesis, Jaffe and colleagues (122-127) delineated the pathways of folate-related metabolism in a filaria-susceptible strain of Aedes aegypti mosquitoes and that a characteristic change occurred in the activity of certain folate-related enzymes when the mosquitoes became infected with the parasites. The activities of dihydrofolate reductase, serine hydroxymethyltransferase, N^5,N^{10}-methyleneTHF reductase and methionine synthetase markedly increased, whereas that of N^{10}-formylTHFsynthetase correspondingly decreased. This pattern of change suggested that the folate metabolism in infected mosquitoes was being altered to favour the synthesis of N^5-methylTHF which is required for methionine synthesis (126). Jaffe and Chrin (123) therefore hypothesized that the developing filarial larvae might be depleting the arthropod host stores of methionine or N^5-methylTHF, or both, thereby shifting the equilibrium of the folate coenzymes towards the synthesis of either or both of these compounds.

Against this background it was proposed to investigate the metabolism of methionine in filariae; delineating the metabolic pathways, identifying the enzymes subserving the various steps and characterizing the key enzymes. It was expected that knowledge emerging from these studies would deepen our understanding of the metabolism of filarial parasites. It might also help to assess the suitability of methionine metabolism as a potentially exploitable target for antifilarial chemotherapy.

Chapter 2

MATERIALS AND METHODS

MATERIALS

[8-^{14}C]Adenosine (sp. radioactivity 57.7 mCi/mmol), S-adenosyl-L-[CH_3-^{14}C]methionine (62 mCi/mmol), L-[^{35}S]methionine (303 mCi/mmol), L-[CH_3-^{14}C]methionine (57.2 mCi/mmol), [CH_3-^{14}C]choline (59 mCi/mmol) and [CH_3-^{14}C]-N^5-methyltetrahydrofolate (58 mCi/mmole) were obtained from Amersham Corp. Arlington Heights, IL. Minimum essential medium Eagle (MEME) with Earle's balanced salts (BSS) was from Microbiological Associates, Bethesda, MD. Thin layer chromatographic plates were purchased from Uniplate; Analtech Inc., Newark, DE. Suramin was kindly provided by Dr. G. Lammler, Justus Liebig Univ., Giessen, Fed. Repub. of Germany; diethylcarbamazine was from Lederle Laboratories, Pearl River, NY. L-Homocysteine was prepared from L-homocysteine thiolactone as described by Mudd et al. (128). Molecular weight calibration kit was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, NJ. Unless otherwise indicated all other chemicals were from Sigma Chemical Co., St. Louis, MO., and they were analytical grade.

Dogs infected with D.immitis and gerbils with B.pahangi were from Dr. John W. McCall, Univ. of Georgia, Athens, GA.

METHODS

A. Delineation of Methionine Metabolic Pathway in Filariae: Adult female B.pahangi were removed from the peritoneal cavities of the gerbils, washed in 0.9% saline solution, blotted carefully, and groups of two were placed in 2 ml of sterilized MEME and BSS. To each of five

such vials was added 15.16 uCi [^{35}S]methionine, and the worms were incubated at 37°C for 24 h in a thermostatically controlled water bath. Thereafter, the worms were washed quickly with 100 ml of 0.9% saline solution, blotted dry, weighed and homogenised in a mini Potter-Elvehjem homogeniser with 0.5 ml of 1.5M perchloric acid for 1 h. A modified version of the method of Shapiro and Ehninger (129) was used to process the worms. The homogenate was centrifuged at 30,000 xg for 30 min in a Sorval RC-2B refrigerated centrifuge. Metabolites of [^{35}S]methionine present in the supernatant fraction were separated with the appropriate carriers by thin layer chromotography (TLC) on silica gel F plates using n-butyl alcohol-acetic acid-water (12:3:5 v/v/v) as solvent. AdoMet and AdoHcy were detected under UV light (at 254) and the others by spraying the chromatoplate with 0.2% ninhydrin in n-butyl alcohol and heating in an oven at 110°C for about 10 min. Spots that co-chromatographed with standards were scraped off, and the radioactivity therein was determined.

B. Preparation of Extract: Adult D.immitis were quickly removed from the heart and the pulmonary artery of a euthanised infected dog and were placed in phosphate-buffered isotonic saline (pH 7.0) containing 0.5% glucose; the worms were then repeatedly washed in this saline solution to remove blood clots. They were sexed, blotted and either immediately processed for enzyme assay or transferred into the appropriate medium for in vitro experiments.

For enzymatic studies the worms were minced with scissors and homogenised at 0°C in a Potter-Elvehjem homogeniser in 4 ml of 0.01M Tris-HCl buffer (pH 7.4) containing 0.25M sucrose, 0.01M $MgCl_2$

and 0.001M 2-mercaptoethanol (130) per gram of worm. The resulting homogenate, to which fine glass beads (3 parts of homogenate:1 part of glass beads) were added, was sonicated at -12°C for four 20 sec intervals at a power output of 60W using a Sonicator Cell Disruptor, Model W-220F (Heat Systems-Ultrasonics, Inc, Plainview, Long Island, NY). The sonication step was deleted in studies dealing with intracellular enzyme localisation. The final homogenate was spun for 30 min at 30,000 xg at 2°C. The supernatant fraction was dialysed overnight against the same buffer but without sucrose at 4°C. Unless needed for immediate enzyme assay, the dialyzed extract was stored at -70°C.

C. Enzyme Assays:

(a) N^5-MethylTHF:homocysteine S-methyltransferase (EC 2.1.1.13): The assay was according to the method of Mudd et al. (131) as modified by Jaffe and Chrin (126).

(b) Betaine:homocysteine S-methyltransferase (EC 2.1.1.5): The method employed was as described by Jaffe and Chrin (126).

(c) ATP:methionine S-adenosyltransferase (EC 2.5.1.6): The assay procedure was a modification of the method of Chiang and Cantoni (132). The incubation mixture contained 84 mM of Tris-HCl, pH 7.8; 40 mM of KCl; 20 mM of $MgCl_2$; 1 mM of KCN; 10 mM of ATP; 5 mM of unlabelled methionine; 50 nCi of [CH_3-^{14}C]methionine; 1 mM of 2-mercaptoethanol; 100 ul of extract and water to a final volume of 250 ul. The reaction mixture was incubated at 30°C for 60 min and the reaction was terminated by adding 0.05 ml of 40% (v/v) trichloroacetic acid. After allowing to sit on ice for 10 min the precipitate was spun down. Twenty ul of the

supernatant fraction was applied on to precoated silica gel G TLC plates (250 um). The chromatogram was developed in 1-butanol-water-acetic acid (12:5:3 v/v/v). On spraying the plate with ninhydrin the spot that co-chromatographed with authentic AdoMet was scraped off and counted for radioactivity.

(d) S-Adenosylmethionine (AdoMet):homocysteine S-methyltransferase (EC 2.1.1.10): The method of Mudd (134) was modified and was used to assay the transmethylase. Contained in a total volume of 250 ul were 0.1M potassium phosphate buffer, pH 7.5; 2 mM homocysteine; 62.5 nCi S-adenosyl-[CH_3-^{14}C]methionine and 100 ul of extract. The mixture was incubated at 37°C for 60 min and the reaction was stopped with 50 ul of 1.84M perchloric acid. The subsequent treatment was as given in the procedure for ATP:methionine S-adenosyltransferase. The methionine spot was scraped off and counted for radioactivity.

(e) S-Adenosylhomocysteine (AdoHcy)hydrolase (EC 3.3.1.1): The enzyme was assayed in the reverse direction by following the formation of S-adenosylhomocysteine. The assay system contained the following: 0.05M potassium phosphate buffer, pH 7.0; 0.3 mM homocysteine; 0.4 mM unlabelled adenosine; 50 nCi [8-^{14}C]adenosine; 3 mM mercaptoethanol and 100 ul of extract. The total volume of the reaction mixture was 250 ul. Incubation was carried out at 37°C for 30 min after which the reaction was stopped with 0.05 ml cold 1.84M perchloric acid. Later treatment was as described above except that silica gel GF. was used in place of silica gel G. The spot containing AdoHcy was detected under UV light, scraped off and radioactivity therein determined.

(f) Cystathionine-β-synthase (EC 4.2.1.22): The reaction mixture consisted of the following: 40 mM potassium phosphate buffer, pH 8.0; 8 mM homocysteine; 2.5 mM serine; 0.12 mM pyridoxal phosphate; 100 ul of extract and water to a final volume of 0.3 ml. After 60 min incubation at 37°C the reaction was stopped by the addition of 0.7 ml ninhydrin reagent which was composed of 150 mg ninhydrin, 15 ml glacial acetic acid and 5 ml phosphoric acid (133). The colour was developed by heating the reaction mixture at 100°C for 5 min; the tubes were then cooled on ice and the absorbance at 455 nm read after 10 min against a minus-homocysteine blank.

(g) Cystathionine-γ-lyase (EC 4.2.1.15): A total assay volume of 0.5 ml contained 40 mM phosphate buffer, pH 7.5; 0.12 mM pyridoxal phosphate; 2 mM cystathionine; and 100 ul of extract. The mixture was incubated for 60 min at 37°C. The reaction was terminated by rapid cooling on ice for 5 min after which 0.5 ml of 10 mM dithiothreitol and 10 ul of 2M NaOH (134) were added. The reaction was allowed to continue at room temperature for 30 min. Afterwards 2 ml of freshly prepared ninhydrin reagent (133) was added and the tubes were heated for 5 min at 100°C. After allowing to cool on ice for 2 min the absorbance at 550 nm was read against a minus-extract blank.

All spectrophotometric readings were conducted using a Beckman Model 25 Recording Spectrophotometer. Radioactivity was assayed by means of liquid scintillation counting of samples in a toluene-based cocktail of composition 6 g 2,5-diphenyloxazole (PPO), 500 ml absolute ethanol and 1400 ml toluene using Packard Tri-Carb spectrometer Model 3002. Internal standardization was used to correct samples for quenching.

Protein concentration was estimated according to the method of Lowry et al. (135) with bovine serum albumin as standard.

Enzyme activity was expressed as micromole of product formed/hour/mg protein under the particular assay condition. The amount of cystathionine and cysteine formed was found by extrapolation from calibration curves.

D. Metabolic Fate of AdoMet in Filariae: To determine the fate of AdoMet, two groups of 5 female D.immitis were placed in 50 ml of the standard medium, containing 350 units/ml streptomycin, to which was added 25 uCi of L-[CH_3-^{14}C]methionine. After incubation at 37°C for 6 h the worms were removed, washed with six 50 ml aliquots of isotonic saline, blotted, weighed and stored at -70°C until required for analysis.

(a) Extraction of Protein: The acid soluble fraction was obtained by homogenising the worms twice with 1.5 ml of 1M perchloric acid at 0°C and centrifuging the homogenate at 30,000 xg for 30 min at 2°C. The pellet was washed three times with the same acid. Protein was extracted by the method of Ogur and Rosen (136). To the protein fraction was added an equal volume of methanol and the sample rotary-evaporated to dryness. The residue was resuspended in 4 ml of methanol and again dried. The dried sample was hydrolyzed in 2 ml of 6M HCl for 40 hr at 110°C. The hydrolysate was dried under reduced pressure and the residue was dissolved in 1.0 ml of deionised water. Methylated amino acids were separated by ascending paper chromatography on Whatman #1 filter paper with pyridine-acetone-3M NH_4OH (50:30:25 by vol.) (137). The areas corresponding to methionine, N-ϵ-methyllysine, 3-methylhistidine and DL α-methylhistidine were located by ninhydrin and the radioactivity therein was determined.

(b) Extraction of Nucleic Acids: DNA and RNA were extracted essentially according to the procedure of Huberman and Sachs (138). One hundred and twenty-five milligrams of adult D.immitis were homogenised with 4 ml of 0.01M Tris-HCl buffer, pH 7.4, containing 0.01M EDTA, 0.01M NaCl and 1% sodium dodecyl sulphate. The resulting homogenate was extracted with equal volume of phenol, and the emulsion obtained centrifuged at 1,200 xg for 5 min. The upper aqueous phase was extracted with an equal volume of phenol-chloroform (1:1 v/v) and the nucleic acids precipitated overnight at -20°C with 2 volumes of absolute ethanol after addition of 0.2M NaCl. After spinning at 2,300 xg for 45 min. the precipitate obtained was dissolved in water and 2M LiCl and the RNA precipitated overnight at 4°C. The precipitate collected at 17,000 xg was dissolved in 0.05M Tris-HCl (pH 6.7), 25mM KCl and 2.5mM $MgCl_2$ (TKM). The DNA remaining in the LiCl was precipitated overnight at -20°C with 2 volumes of absolute ethanol, centrifuged at 12,000 xg for 30 minutes and dissolved in 1.5mM NaCl and 0.15mM sodium citrate, pH 7.2 (SSC). The RNA and DNA solutions were incubated for 90 minutes at room temperature with 50ug/ml DNase in TKM and RNase in SSC respectively. Afterwards the solutions were deproteinized by consecutive extractions with equal volumes of chloroform-phenol (1:1 v/v) and chloroform. The nucleic acids were precipitated overnight at -20°C with 2 volumes of absolute ethanol after addition of 0.2M NaCl and then dissolved in 1.0ml of SSC. The absorbance at 260nm was taken against a solvent blank.

The isolated DNA and RNA were incubated at 37°C for 1 hour with 0.1ml of 50 ug/ml DNase, RNase or pronase to remove any further contaminating nucleic acids and proteins. The radioactivities of the recovered acid-soluble DNA and RNA after denaturation with 0.2ml 10% TCA in the presence of 0.1ml 0.5% bovine serum albumin were determined.

(c) Extraction of Lipids: While still frozen, a batch of filariae was manually crushed in a glass homogeniser, and then total lipids were extracted twice with each of the following solvents in the order given: a) 100% methanol; b) 60% methanol:40% chloroform, and c) 40% methanol:60% chloroform. Extractions were carried out at 0°C, each successive extract was centrifuged under refrigeration at 1000 xg for 10 min, the pellets were reextracted, all supernatant fractions were pooled, and finally evaporated to dryness under reduced pressure. Phospholipids were eluted with methanol from a column of silicic acid (100-300 mesh) and celite (diatomaceous earth) (2:1 w/w), following the method of Burt et al.(139). The eluted phospholipids were rotary evaporated to dryness, resuspended in cyclohexane and a known aliquot (100 ul) removed for measurement of radioactivity. The phospholipid fraction was analyzed by thin layer chromotography on silica gel H with chloroform-methanol-acetic acid-water (25:15:4:2 v/v/v/v) (140) and with 5 mg/ml of the following dissolved in methanol-chloroform (1:2 v/v) as standards: synthetic lecithin, lysolecithin, sphingomyelin, dimethylphosphatidylethanolamine and phosphatidylethanolamine. The lipids were visualized by exposing the TLC plate to iodine vapour. The TLC plate was then divided into 1-cm squares, spots scraped off and the radioactivity in each determined. Another TLC plate was developed, photographed and an autoradiogram prepared.

Autoradiography: Upon the disappearance of the iodine the chromatogram was placed in opposition to a sheet of Kodak X-Omat-RP film (Eastman Kodak, Co., Rochester, NY) and left in a light proof x-ray cassette holder in the dark. At the end of a 2-week exposure period the film was developed.

Chapter 3

RESULTS

A. Biosynthesis of Methionine by Filariae

Experimental findings indicated that at least adult filariae are unable to synthesise methionine de novo and instead probably rely upon an exogenous source of this amino acid. Repeated attempts to detect the presence of vitamin B12-dependent and independent methionine synthesising enzymes proved fruitless (Table 3). Moreover, incubation of adult B.pahangi with N^5-(CH_3- ^{14}C)methylTHF ($^{14}CH_3THF$) revealed that there was no incorporation of radioactivity into methionine, as compared with the substantial counts in methionine extracted from mammalian cells (L 1210) incubated under identical conditions (Table 1). The results confirm those reported by Jaffe et al (142).

Table 1: Biosynthesis of Methionine by Filariae

Female B.pahangi were incubated with 20 uCi of $^{14}CH_3THF$ for 2 and 24 h. At the end of the incubation period the worms were washed with 0.9% saline and processed by the procedure of Schmidt and Thannhauser (141). The Trichloroacetic acid (TCA)-soluble fraction was analyzed chromatographically for methionine by the method of Jaffe and Chrin (126). Incubation of L 1210 under identical conditions was undertaken for comparison.

	COUNTS/MINUTE	
SYSTEM	2 h	24 h
B.pahangi	0	0
L 1210	1526	4130

B. Ability of Filarial Worms to Metabolize Methionine

To test for the ability of filarial worms to metabolize methionine adult B.pahangi were incubated in vitro in the presence of (^{35}S) methionine. The results are given in Table 2.

Table 2: Ability of Filarial Worms to Metabolize Methionine

Adult female B.pahangi were incubated in the presence of (^{35}S) methionine. After 24 h the worms were homogenized in perchloric acid and the supernatant fraction obtained analyzed chromatographically for metabolites of methionine. Details of experimental procedure are given in Materials and Methods section.

	cpm/mg worm
Methionine	149
S-Adenosylmethionine	651
S-Adenosylhomocysteine	792
Homocysteine & cysteine	388

There was incorporation of radioactivity into AdoMet, AdoHcy, homocysteine and cysteine, demonstrating that filariae possess the enzymatic machinery for metabolizing methionine analogous to that found in prokaryotes and other eukaryotes. The enzymes involved were subsequently detected in filarial extracts and their specific activities are shown in Table 3. Prior to determining specific activities linearity was established between enzyme activity, duration of assay, and protein concentration. Relatively high levels of S-adenosylhomocysteine hydrolase and the transsulphuration enzymes were found, though generally the activity of the methionine metabolizing enzymes in filariae under these experimental conditions was low.

Table 3: Specific Activities of Some Filarial Methionine Metabolizing Enzymes

Dialysed supernatant fraction of freshly obtained female D.immitis was used in the various enzyme assays. For details on extract preparation and assay systems, see the section on Materials and Methods.

Enzyme	Specific Activity[a]
ATP:methionine S-adenosyltransferase	0.02
S-Adenosylmethionine:homocysteine S-methyltransferase	0.11
S-Adenosylhomocysteine hydrolase	5
Cystathionine-β-synthase	225
Cystathionine-γ-lyase	7
Methionine synthetase	0
Betaine:homocysteine S-methyltransferase	0

[a] nmole product formed/h /mg protein

C. Partial Characterisation of S-Adenosylmethionine:Homocysteine S-Methyltransferase

Despite the absence of vitamin B12-dependent and -independent methionine synthesis in filarial worms, the detection of AdoMet:homocysteine S-methyltransferase in filarial extracts suggested that these parasites could meet some of their methionine requirement by way of this enzymatic reaction. An attempt was made to partially characterize this enzyme.

In preliminary experiments it was found that the activity of this enzyme was slightly higher in supernatant fractions of extracts maintained at 50°C for 5 min; consequently this heat treatment was used to partially purify the enzyme. A summary of the purification procedure is shown in Table 4. A five-fold increase in activity over the crude extract was achieved, with the yield almost unchanged.

Table 4: Partial Purification of Filarial AdoMet:Homocysteine S-Methyltransferase

Purification Step	Volume (ml)	Total Acct. (*)	Protein Conc. (mg/ml)	Sp.Act. (units/mg protein)	Yield (%)	Deg. of Purification
Crude extract	21	10.3	4.45	0.11	100	1
50°C for 5 min	15	9.7	1.25	0.52	94	5

*nmole of methionine produced per hour.

(a) Intracellular Localization of AdoMet:Homocysteine S-Methyltransferase

Differential centrifugation studies coupled with deoxycholate treatment of the resulting pellets indicated that AdoMet:homocysteine S-methyltransferase is exclusively cytosolic (Table 5).

Table 5: Intracellular Localization of AdoMet:Homocysteine S-Methyltransferase

Homogenate of adult female D.immitis was subjected to differential centrifugation. The activity of the methyltransferase in the various fractions was determined under optimum assay conditions.

Fraction	d.p.m. recovered in methionine
Nuclear	0
Mitochondrial	0
Microsomal	0
Supernatant (cystosol)	1909

(b) Dependence of Enzyme Activity on Time and Enzyme Concentration:

The activity of AdoMet:homocysteine S-methyltransferase was linear with time for up to 120 min, after an initial lag period of 30 min (Fig.5). The rate of production of methionine was also directly proportional to the amount of enzyme present in the incubation mixture (Fig. 6).

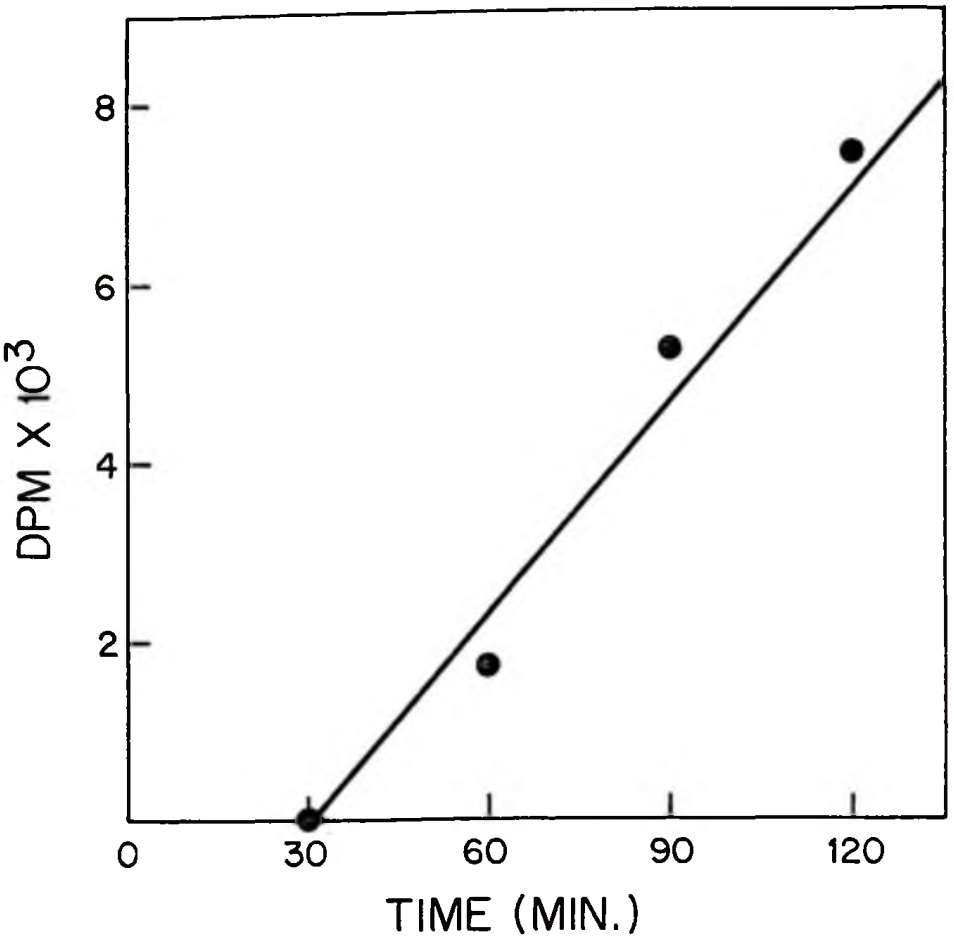


Figure 5

Time-Course of Formation of Methionine by

AdoMet:Homocysteine S-Methyltransferase

Different reaction mixtures were set up. At the end of the incubation period the reaction was terminated with perchloric acid. The radiolabelled methionine formed was separated chromatographically and estimated by liquid scintillation spectrometry. Details of the procedure are found in the Materials and Methods.

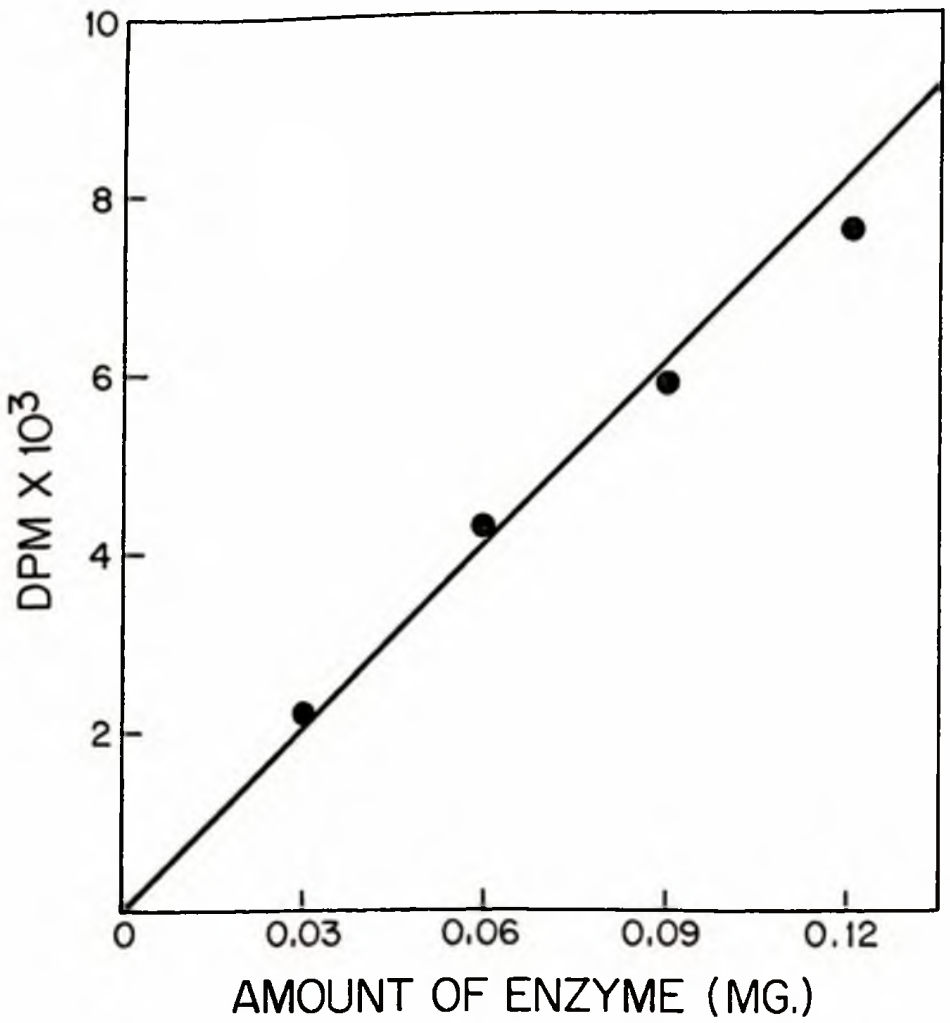


Figure 6

Formation of Methionine by AdoMet:Homocysteine S-Methyltransferase as a Function of Enzyme Amount

Different volumes of the enzyme preparation (2 mg/ml) were incubated as described in the standard assay. The amount of product formed was determined by liquid scintillation counting.

(c) Effect of pH and Ionic Strength on Enzyme Activity: The pH-activity profile in phosphate buffer gave a single sharp peak at pH 8.0 (Fig.7). However, because of the instability of AdoMet in alkaline medium (195-198) the effect of ionic strength was investigated at pH 7.5, varying the concentration of the buffer from 25-200 mM. The activity of the enzyme was optimal at around 100 mM (Fig. 8).

(d) Effect of Temperature on Enzyme Activity: Fig. 9 shows the effect of temperature on AdoMet:homocysteine S-methyltransferase. The activity of the enzyme increased up to 50°C, after which it declined rapidly.

(e) Requirement of Assay: Table 6 indicates the requirements for the filarial AdoMet:homocysteine S-methyltransferase reaction. No methionine was produced when the extract was omitted. In the absence of homocysteine a limited amount of methionine was formed, even after extensive dialysis. Of a variety of thiol compounds tested in place of mercaptoethanol, cysteine gave the highest rate of product formation; dithiothreitol (DTT) and reduced glutathione (GSH) were slightly inhibitory.

Table 6: Requirement of Assay of Filarial AdoMet:Homocysteine S-Methyltransferase [b]

The complete assay system is described in the Materials and Methods.

	Nanomole methionine/h /ml
Complete assay mixture	0.228
Minus homocysteine	0.017
Minus mercaptoethanol	0.123
" " + cysteine	0.527
" " + dithiothreitol	0.201
" " + reduced glutathione	0.156
" extract	0

[b] Extract was prepared in 0.04M Tris-HCL buffer, pH 7.4

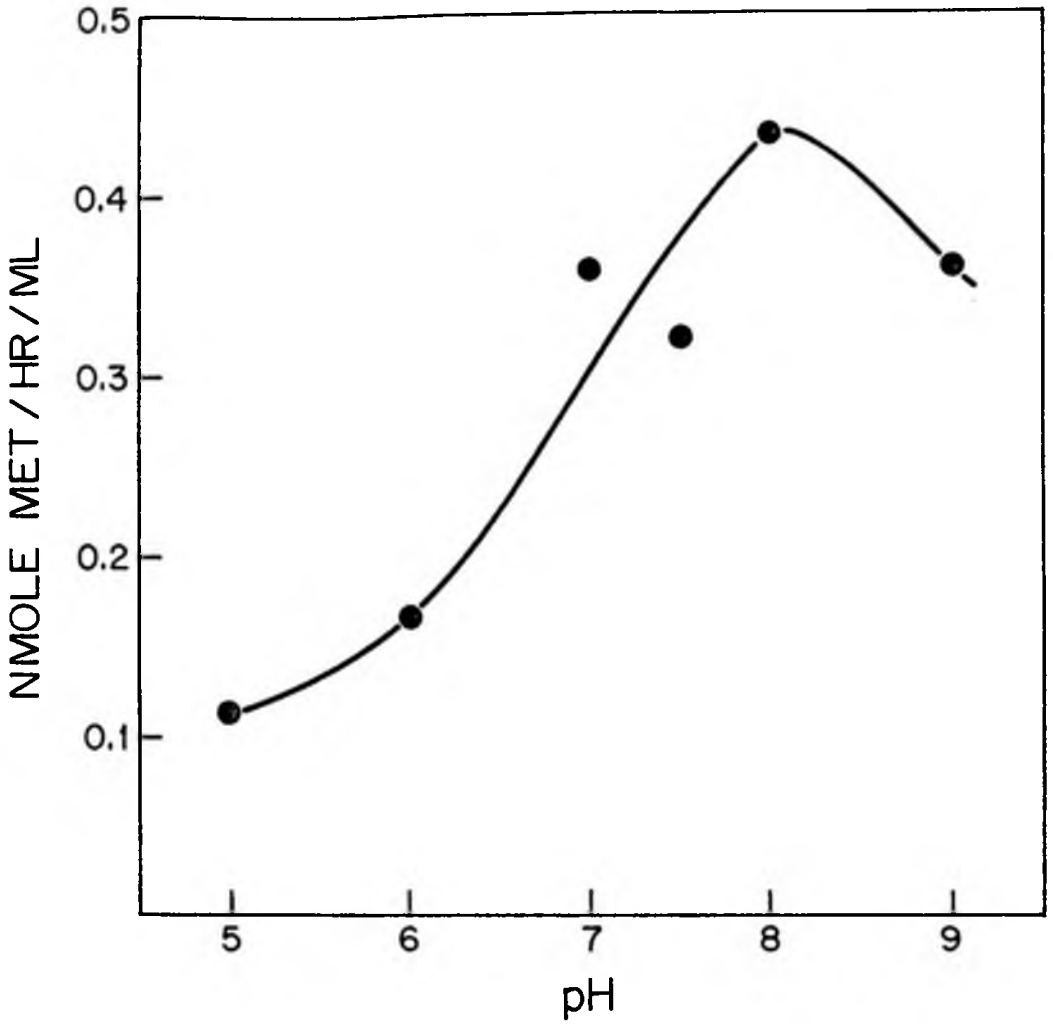


Figure 7

Effect of pH on AdoMet:Homocysteine S-Methyltransferase

The methyltransferase assay was run in 0.1M potassium phosphate buffer of varying pH values. The amount of labelled methionine formed was determined as described in the standard assay.

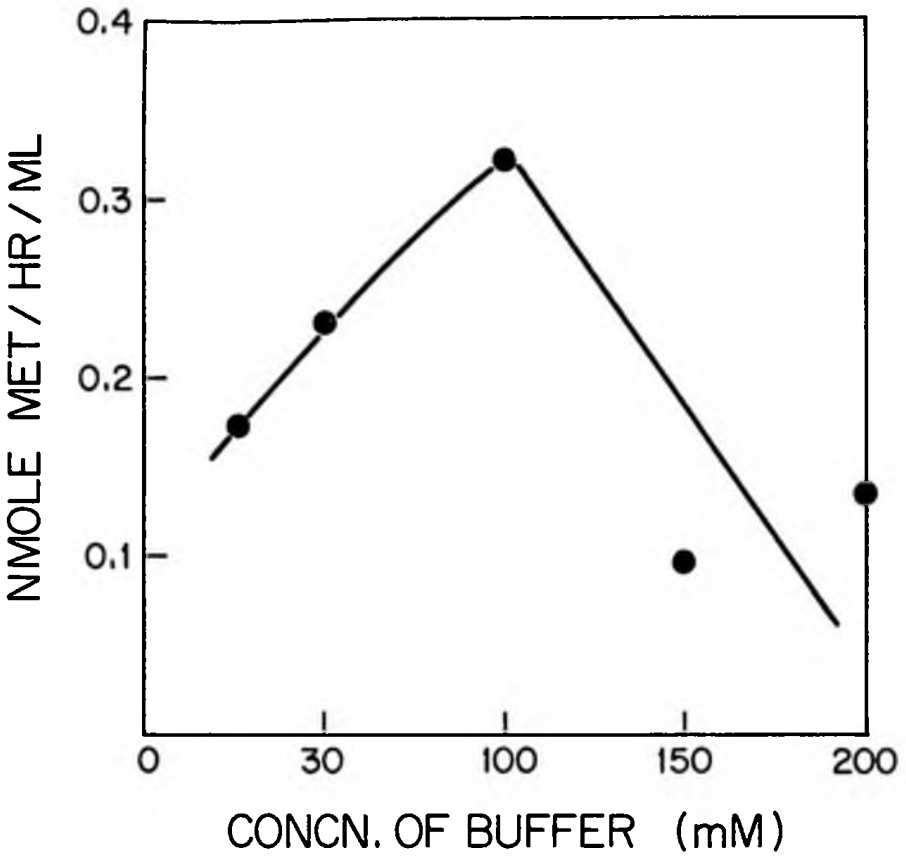


Figure 8

Effect of Ionic Strength on AdoMet:Homocysteine S-Methyltransferase

The assay was performed in various concentrations of potassium phosphate buffer pH 7.5. The rest of the procedure is as described in the standard assay.

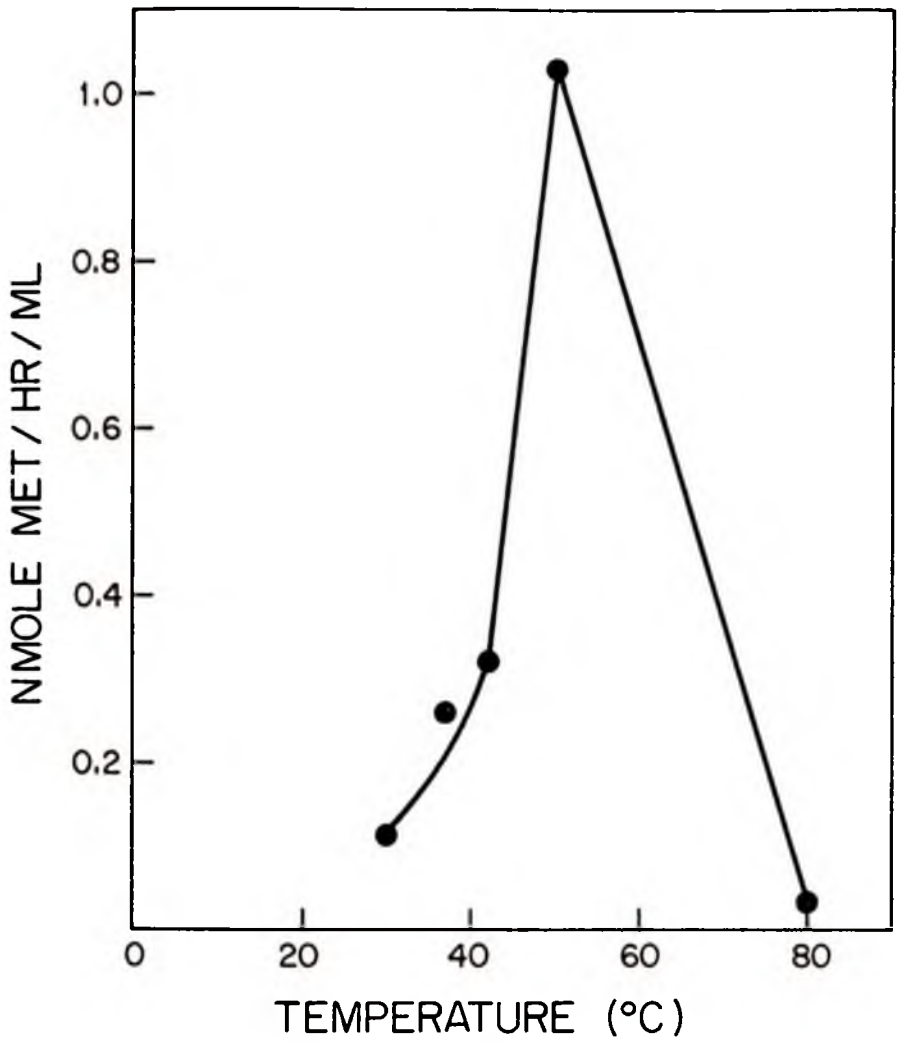


Figure 9

Effect of Temperature on AdoMet:Homocysteine S-Methyltransferase

The enzyme assay was run at various temperatures under conditions given in the standard method.

(f) Effect of Divalent Metal Ions and EDTA: All the metal ions studied activated the methyltransferase (Table 7) The alkaline earth metal ions, Mg^{++} and Ca^{++}, were more effective than the transition divalent metals, Mn^{++}, Zn^{++} and Co^{++}.

Table 7: Effect of Metal Ions on Filarial AdoMet: Homocysteine S-Methyltransferase(b)

The enzyme was incubated with various divalent metal ions and the enzyme activity was measured as in the standard assay conditions.

10^{-3}M Cation	Nanomole methionine formed/h /ml
None	0.225
Mg^{++}	0.551
Ca^{++}	0.428
Mn^{++}	0.299
Zn^{++}	0.285
Co^{++}	0.320

As a result of the above findings the effect on the methyltransferase of EDTA was investigated; EDTA concentrations greater than 10^{-5}M were found to be inhibitory (Table 8).

Table 8: Effect of EDTA on Filarial AdoMet: Homocysteine S-Methyltransferase(b)

The Enzyme was incubated with different concentrations of EDTA under normal assay conditions.

EDTA Concn (M)	Nanomole methionine formed/h /ml
None	0.396
10^{-5}	0.450
10^{-4}	0.263
10^{-3}	0.186

(g) Kinetic Studies: The partially purified enzyme preparation exhibited Michaelis-Menten kinetics with respect to both homocysteine and AdoMet. The kinetic constants obtained by the double-reciprocal plot method of Lineweaver and Burk (143) are found in Table 9.

Table 9: Kinetic Parameters of Filarial AdoMet: Homocysteine S-Methyltransferase

The values of the apparent Km and Vmax at pH 7.5 and 37°C were estimated from Figures 10 and 11.

Substrate	Vmax	Km (uM)
AdoMet	0.59	8
Homocysteine	0.39	900

The value of Vmax is expressed as nmole methionine formed/hr/ml.

(h) Effect of Inhibitors:

The effect of the standard antifilarial drugs, DEC and suramin, on the activity of AdoMet: homocysteine S-methyltransferase was investigated. DEC and suramin inhibited this filarial enzyme by 32% and 67% respectively, at a concentration of 0.1 mM. At the same level of AdoHcy there was no effect.

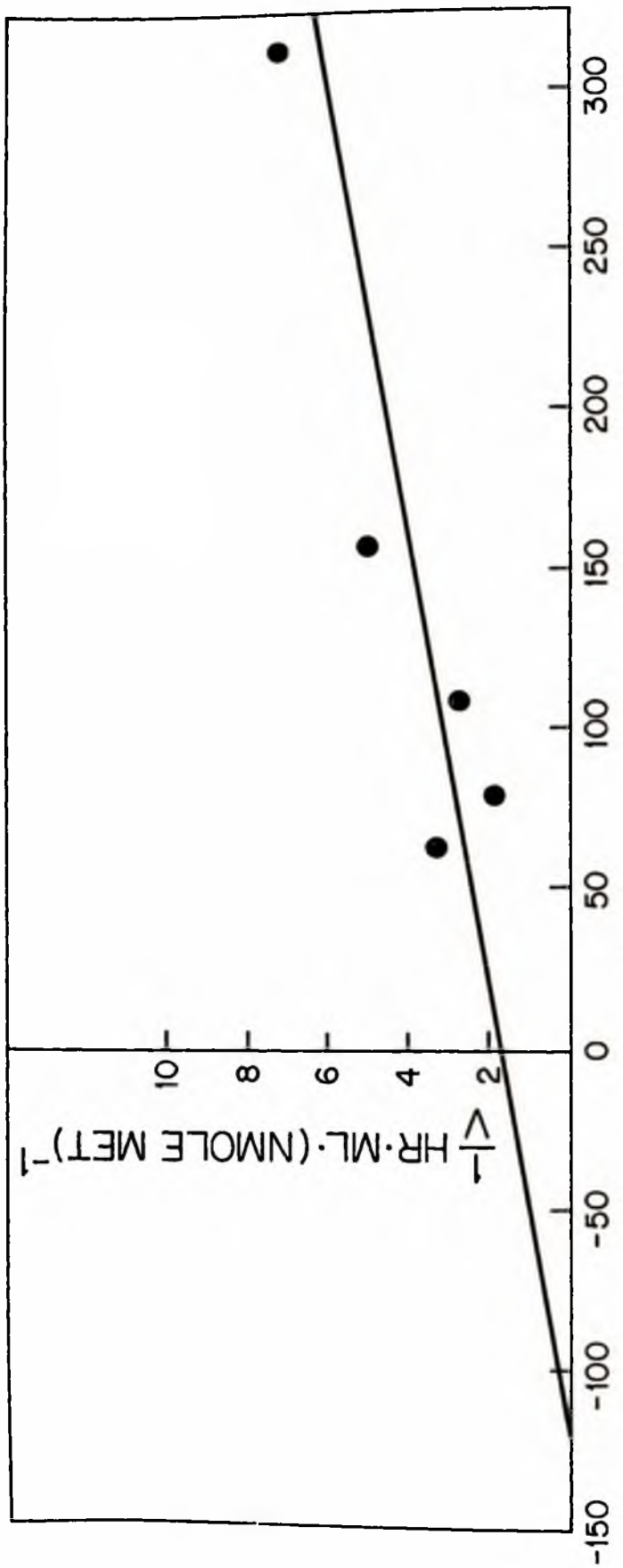


Figure 10

Lineweaver-Burk Plot of AdoMet Keeping Homocysteine Concentration Constant

The standard assay system was employed except that the concentration of AdoMet was varied keeping that of homocysteine at 2mM.

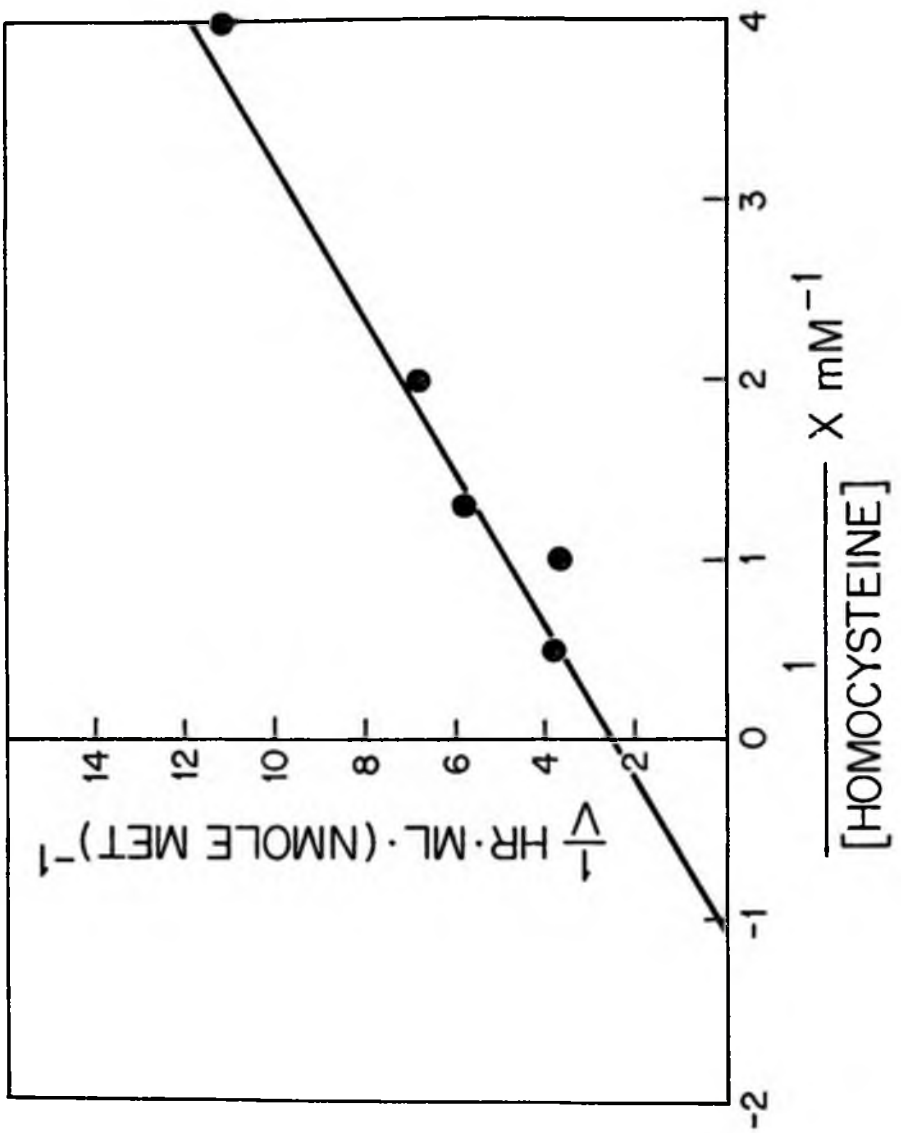


Figure 11

Lineweaver-Burk Plot of Homocysteine Keeping AdoMet Concentration Constant

The standard assay system was employed except that the concentration of homocysteine was varied holding that of AdoMet constant at 0.016mM.

D. Metabolic Fate of AdoMet in Filariae: Because of the significance of AdoMet-dependent methylations in cell function (144-146), identification of other methyl acceptor compounds in filariae was undertaken. This was preformed by incubating adult female D.immitis in a medium containing (CH_3-^{14}C)methionine to induce these filariae to synthesise radiolabelled AdoMet. The distribution of this radioactivity among the various filarial fractions was as shown in Table 10.

Table 10: Distribution of Radioactivity after Incubating Adult D.immitis in the Presence of (CH_3-^{14}C) Methionine

Adult female D.immitis were incubated in (CH_3-^{14}C)methionine for 6h. Afterwards the parasites were processed and the radioactivity in the various fractions determined by liquid scintillating counting. Details of the experimental procedures are described in the Materials and Methods.

Fraction	dpm	%
Acid-soluble	145136	42.4
Total lipids	71369	20.8
Phospholipids	68941	20.1
Neutral lipids	1000	0.3
Protein	126192	36.8
Nucleic acid	0	0

It can be seen that the total lipid fraction contained 21% of the label incorporated by these parasites, most of the counts being found in the phospholipid fraction. TLC of the filarial phospholipid fraction (Fig.12) indicated that the radiolabelled phospholipids were dimethylphosphatidylethanolamine, lysolecithin, lecithin and sphingomyelin. The incorporation of radioactivity into dimethylphosphatidyl-ethanolamine was of the same amount as that associated with the other phospholipids. An autoradiograph, however, indicated the presence of sphingomyelin (Fig. 13). The other spot in the autoradiograph was identified as methionine. This is because on another TLC plate a spot with relative mobility close to that of the spot in the autoradiograph had co-chromatographed with authentic methionine. In between the methionine and sphingomyelin spots was a diffuse band of radioactivity. Sphingomyelin as well as lecithin, lysolecithin, phosphatidylethanolamine and phosphatidylinositol could be identified among the phospholipids isolated from adult female D.immitis (Plate 1). The composition of the dog heartworm phospholipids closely resembled that of Setaria cervi, a nematode parasite of water buffalo, Bubalus bubalis (107).

The remaining radioactivity was associated with the protein and perchloric acid soluble fractions; the former accounted for 37% of the total dpm incorporated. Fractionation of the protein hydrolysate indicated radioactivity in methionine and methylated lysine and histidine, and some unknown compounds which ran close to the origin (Fig.14). Methylated arginine might be one of these unknowns as there was a spot which cochromatographed with authentic arginine.

In the present investigation no radioactivity was recovered from the nucleic acid fraction during the six hour period of incubation.

Figure 12

Adult female D.immitis were incubated in (CH_3-^{14}C)methionine for 6h. Afterwards the phospholipids were extracted and characterized chromatographically. Details of the experimental procedure are described in Materials and Methods.

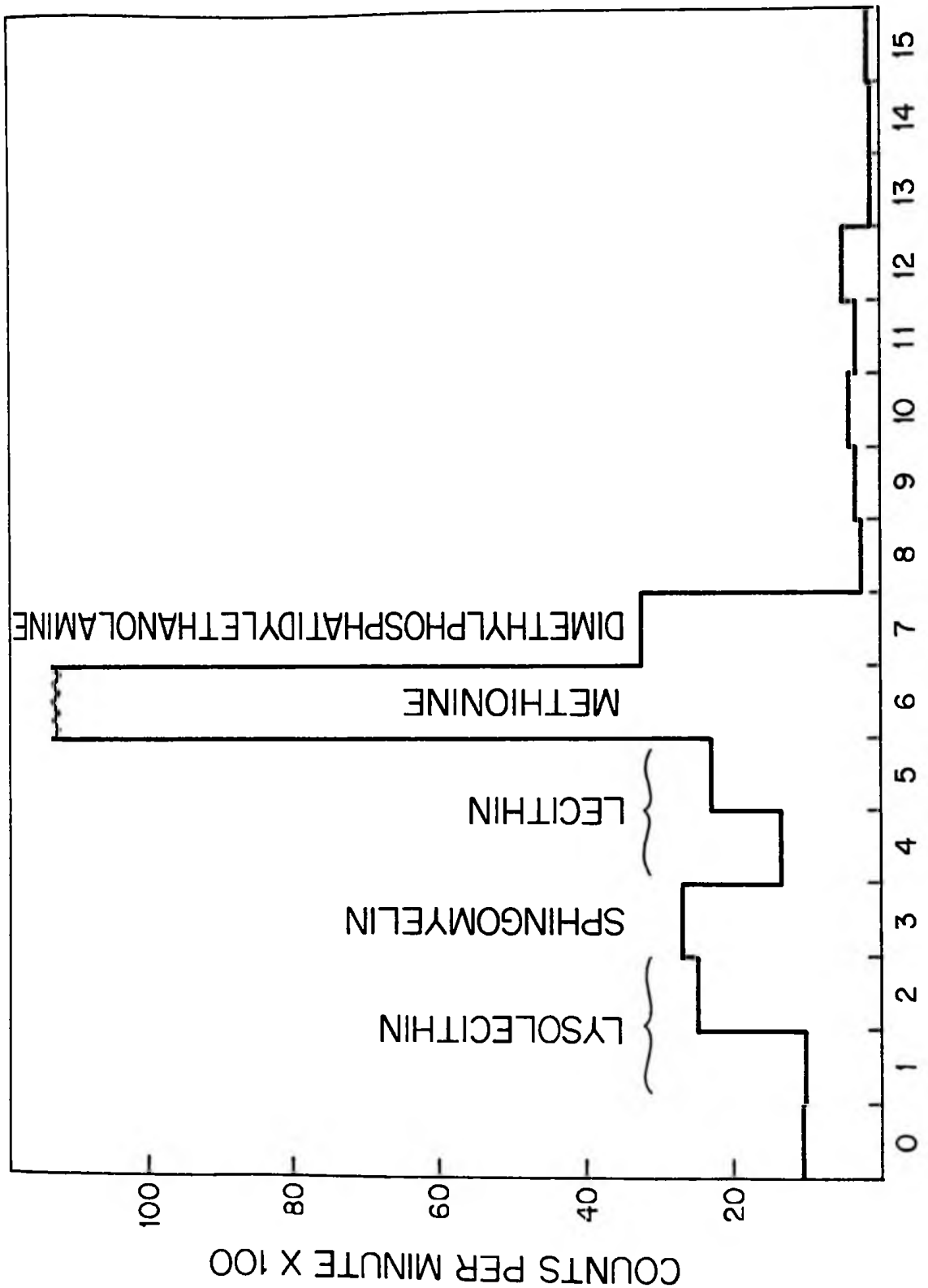


Figure 12

Radioactivity Distribution in Thin Layer Chromatogram of Phospholipids

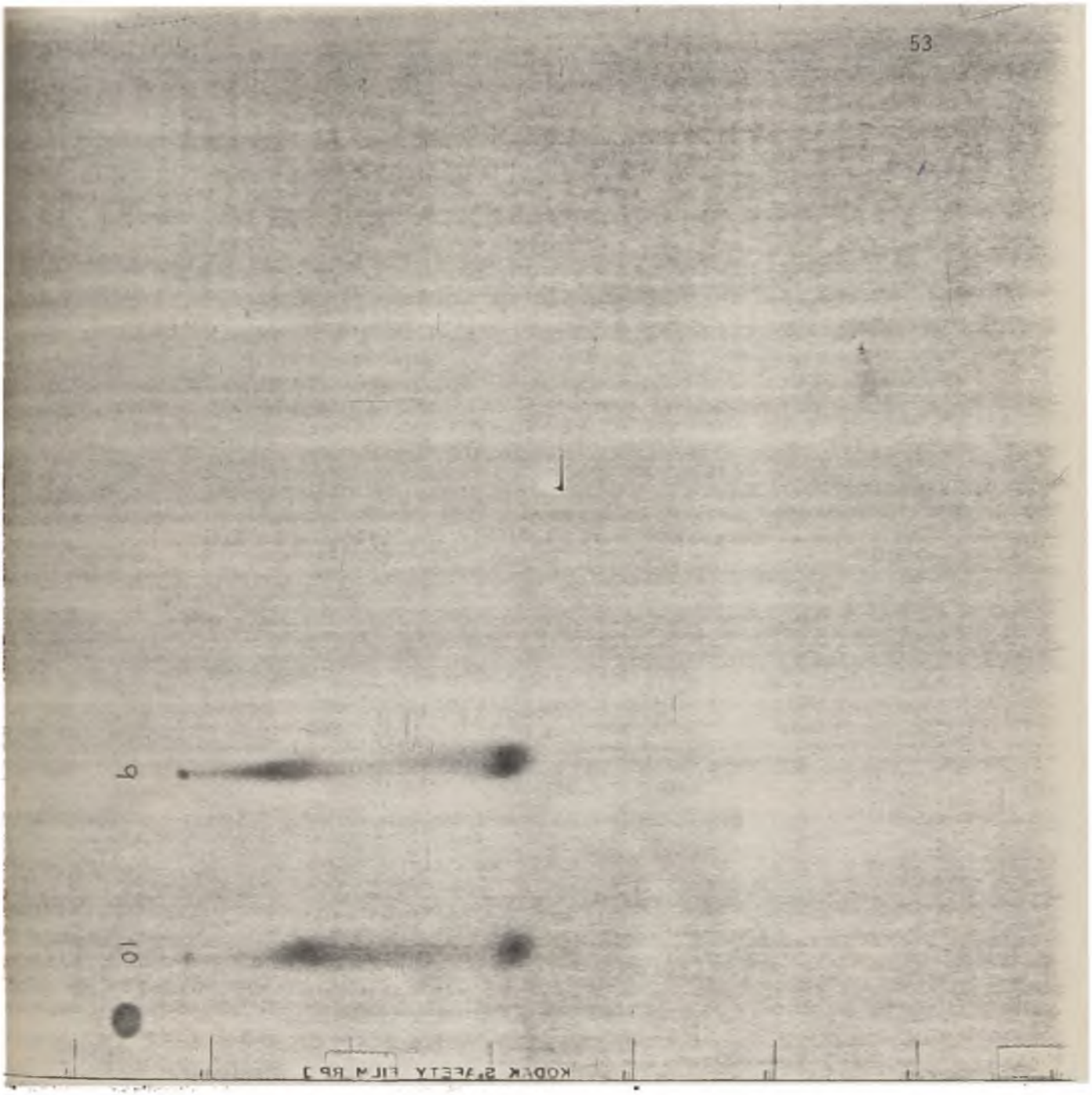


Figure 13
Autoradiogram of Filarial Phospholipids

(See page 54 for composition of 9 and 10)

Figure 13 is an autoradiograph prepared from the chromatograph shown in Plate 1.

PL. 1: Photograph of Iodine-Developed TLC of Filarial Phospholipids

Adult female D.immitis were incubated for 6h in (CH_3-^{14}C)methionine. Isolation of the phospholipid fraction was followed by its resolution by thin layer chromatography. The TLC plate was developed, photographed and later autoradiographed. Experimental details could be found in Materials and Methods.

1. Lecithin
2. Lysolecithin
3. Sphingomyelin
4. Dimethylphosphatidylethanolamine
5. Natural lecithin from egg yolk
6. Phosphatidylinositol
7. Phosphatidylethanolamine
8. Unlabelled phospholipid fraction (cold)
9. Cold + labelled phospholipid fraction
10. Labelled phospholipid fraction + standards (1-7)

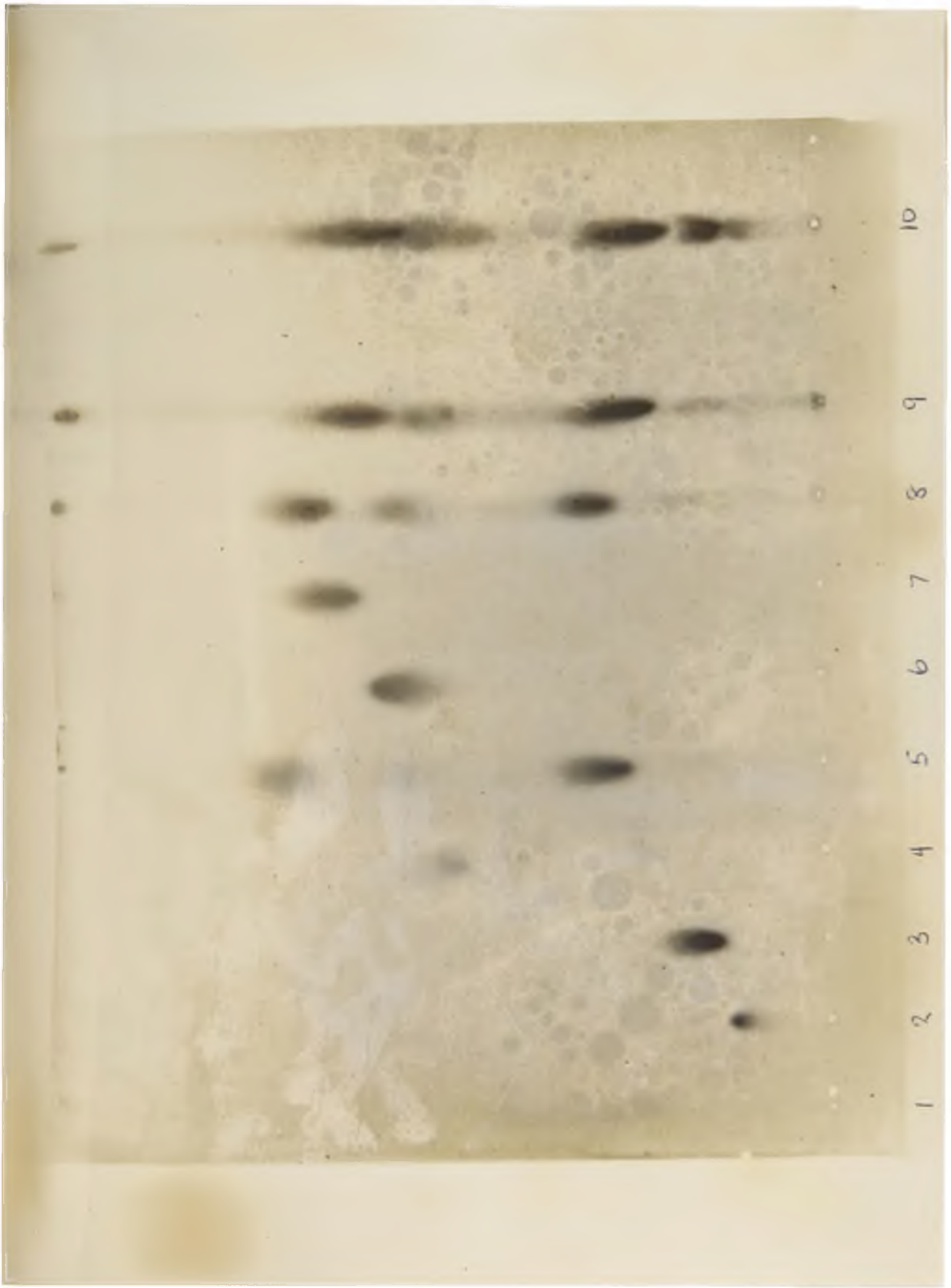


Figure 14

Radioactivity Distribution in Thin Layer Chromatogram of Protein Hydrolyz

Protein fraction extracted from adult female D.immitis incubated for 6h in (CH_3-^{14}C)methionine was hydrolyzed in acid. The hydrolyzate was analyzed chromatographically for methylated amino acids. Details have been described in Materials and Methods.

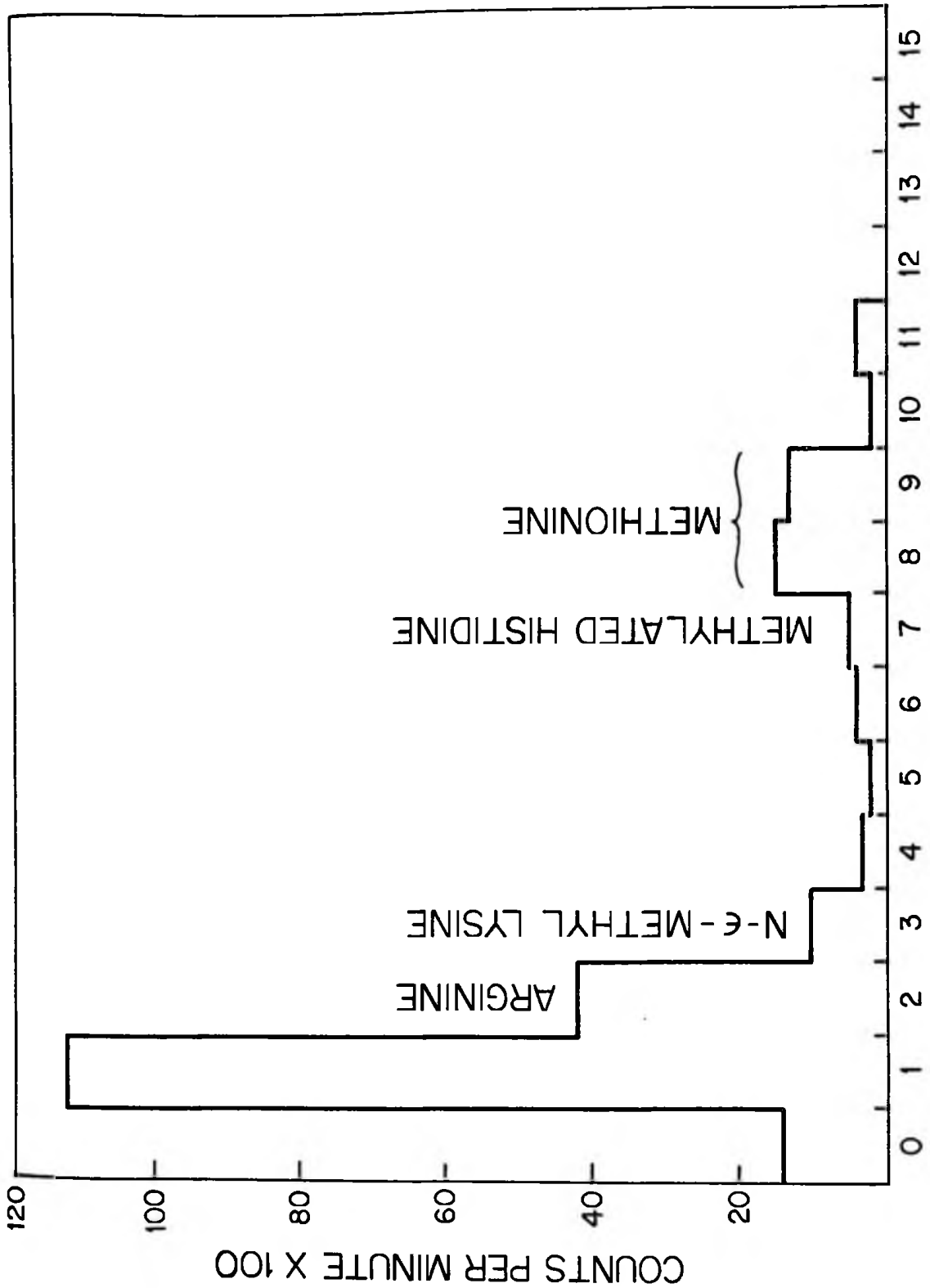


Figure 14

Radioactivity Distribution in Thin Layer Chromatogram of Protein Hydrolysate

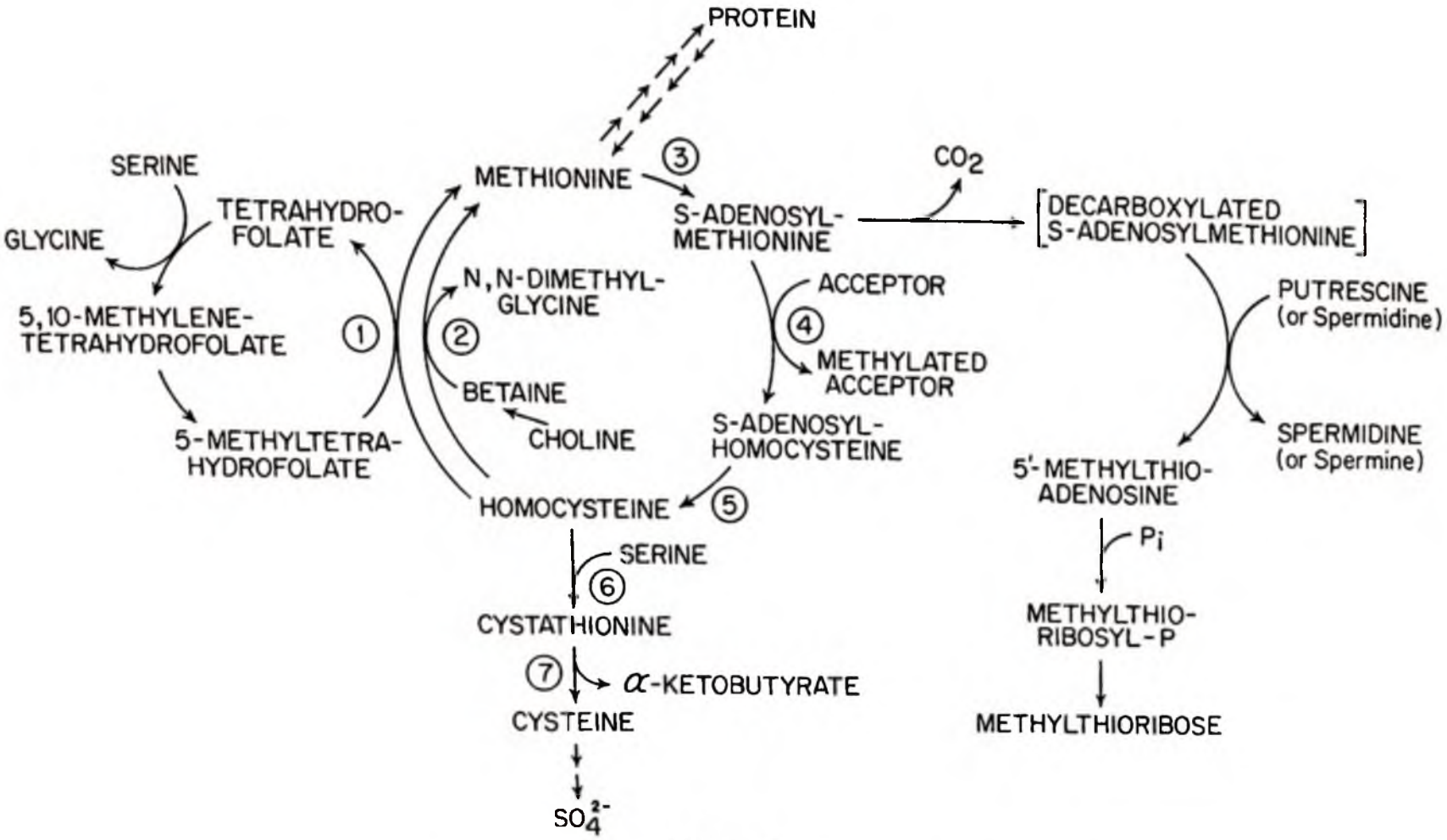


Figure 15

Major Pathways for Methionine Metabolism in Mammals (144)

(1) 5-Methyltetrahydrofolate:homocysteine S-methyltransferase. (2) Betaine:homocysteine S-methyltransferase.
(3) ATP:methionine S-adenosyltransferase. (4) S-Adenosylmethionine:homocysteine S-methyltransferase.
(5) S-Adenosylhomocysteine hydrolase. (6) Cystathionine-β-synthase. (7) Cystathionine-γ-lyase.

Numbers in parentheses represent methionine metabolic enzymes that have been detected in filariae.

Chapter 4

DISCUSSION AND CONCLUSION

A. Folate Metabolism

The role of folic acid in the *de novo* synthesis of purine ribonucleotides and the pyrimidine deoxyribonucleotide, thymidylate, has been amply demonstrated in many biological systems including parasitic nematodes (147,148). Jackson and Siddiqui (149) have shown that *Neoaplectana glaseri*, a parasitic nematode of insects, needs folic acid for reproduction since transformation was halted in the presence of aminopterin and methotrexate, potent inhibitors of dihydrofolate reductase. In addition to the above physiological functions, folic acid is also involved in the biosynthesis of methionine (147) and the provision of the formyl group of N-formylmethionyltransfer ribonucleic acid (150). In view of this, any discussion of methionine metabolism in filariae would be incomplete without considering the role of folate metabolism in filariae. The interrelationship between methionine and folate metabolism is shown in Fig. 15. Jaffe and associates (57,117) have shown that adult filarial worms possess the whole array of folate-related enzymes. Apart from some minor differences, the properties of the enzymes associated with N^5,N^{10}-methylenetetrahydrofolate(CH_2THF), namely serine hydroxymethyltransferase (EC 2.1.2.1), thymidylate synthetase (EC 2.1.2.45), CH_2THF dehydrogenase (EC 1.5.1.5) and CH_2THF reductase (EC 1.1.1.68) are generally similar to the analogous enzymes from mammalian and mosquito sources (122,124,125). Unlike microorganisms where the regulation of CH_2THF dehydrogenase activity appears to control folate and methionine metabolism (150), in the vertebrate and invertebrate systems the reactions of regulatory interest are those catalyzed by CH_2THF reductase and N^{10}-formylTHF dehydrogenase (EC 1.5.1.6) (151,152). These enzymatic steps

ensure regeneration of tetrahydrofolate which otherwise would have been trapped. Methionine, probably through AdoMet, exerts a modulatory influence on these enzymes. In contrast to its activatory effect on formylTHF dehydrogenase (151) methionine causes a negative feedback response on the corresponding reductase (153,154). Consequently on saturation of the folate system with methylTHF any excess one-carbon units will be channelled towards the formylTHF dehydrogenase step and eventually dissipated in the form of CO_2 (151). Regulation of folate metabolism may also be achieved by controlling the uptake mechanisms and the formation of polyglutamates from monoglutamates (151).

Filariae have a relatively highly active NADP-dependent and NADP-independent 10-formylTHF dehydrogenase system compared with its counterpart in mammalian organs such as the liver; the general properties of the analogous filarial and mammalian 10-formylTHF dehydrogenases are similar (58).

B. Methionine Metabolism

(a) N^5-MethylTHF:Homocysteine S-Methyltransferase and Betaine:Homocysteine S-Methyltransferase:

In all biological systems examined so far the thermodynamics of CH_2THF reductase has always favoured the synthesis of methylTHF which is utilized in the formation of methionine (147).

Methionine synthetase which catalyzes this reaction has been isolated from Escherichia coli K-12 and purified to homogeneity, yielding three components (155). These are: M component, methionine synthetase with molecular weight of 186,000 daltons and containing one mole of vitamin B12 per mole of protein; R component, flavine adenine dinucleotide (FAD) flavoprotein with molecular weight of 27,000 daltons; and finally the F component, flavine mononucleotide (FMN) molecular weight 19,400 daltons.
The three components and S-adenosylmethionine are essential for the

biosynthesis of methionine. These accessory factors are utilized to activate the B12 prosthetic group, a process which involves the conversion of the protein bound B12 to methyl-B12 with the simultaneous reduction of cobalt(II)i.e. cob(II)alamin to methylcob(I)alamin (156,157-159). The reaction sequence was derived spectrophotometrically; the spectrum of the M component as isolated showed the cobalt of B12 to be in the +2 valence state. Addition of NADPH (NADH substituted for NADPH but the concentration required was higher owing to its higher Km value) R and F did not alter the spectrum. This is because the reduction of B12(+2) to B12(+1) is so highly endergonic (160). With the addition of S-adenosyl-methionine, enough energy was generated to shift the spectrum to that of methyl-B12 (161,162).

Methylation of the activated methionine synthetase by N^5-methyltetra-hydrofolic acid (N^5-methylTHF) appears to be an intermediate in the synthesis of methionine (163,164). This view was subsequently confirmed (165-167). The methyl group is then donated to homocysteine to yield methionine. Current studies apparently suggest that N^5-methylTHF poly-glutamates are better substrates than N^5-methyl-THF (the monoglutamate form) (168,169). There is also evidence that the same enzyme may be mediating the mono- and poly-glutamate-utilizing reactions.

Methionine biosynthesis in mammals, unlike that in bacteria, requires in addition to S-adenosylmethionine, R, F and M components, transcobalamin-II, a serum protein for transporting B12 across the cell membrane (170-172). Once inside the cells B12 is converted to adenosyl B12 and methylB12, the former predominating (173).

As indicated in Tables 1 and 3 adult filariae appear to be incapable of methionine synthesis from homocysteine even though they possess CH_2THF reductase. Instead they have been found to assimilate methylTHF and oxidise this cofactor to CH_2THF by way of CH_2THF reductase operating in the reverse direction (117). This uncommon property has also been observed in mammalian foetal brain where ^{14}C-labelled CH_3THF was incorporated into the DNA's thymine (144). This metabolic peculiarity suggests that when methyl group synthesis and therefore involvement of folate in AdoMet metabolism is insignificant most of the methionine would be derived exogenously (144). It has been shown that the synthesis of tetrahydroisoquinoline also involves the reversal of the reductase step (144). The formaldehyde generated by this reaction condenses with dopamine in a Pictet-Spengler reaction. Recently Jaffe and Chrin (117) employed this unusual feature to illustrate the ability of adult filariae to synthesize purines de novo.

Du Vigneaud and coworkers (174) observed that in the presence of sufficient dietary choline, folic acid, vitamin B12, serine and glycine, utilization of preformed methyl groups was preferred to de novo-synthesized methyl groups. This observation stimulated the search for (an) alternative B12-independent pathway of methionine synthesis that culminated with the report that in mammals methionine could also be produced in the presence of homocysteine plus choline, betaine or certain methylsulphonium compounds such as dimethylacetothetin, ethylmethylacetothetin and dimethylpropiothetin (175). Thetin-homocysteine methylpherase catalyzed

this reaction. The preferred substrate for the enzyme, dimethy-lacetothetin, has, however, not been detected in nature. A second transmethylating enzyme, betaine:homocysteine methylpherase or transferase with a high affinity for the naturally occurring methyl donor, betaine, was later discovered (176). The livers of all the vertebrates investigated appeared to have a high activity of this transferase (177). Structural-activity studies on the transferase seem to suggest that the carboxylmoiety of betaine is not required for activity while the N-methyl groups are essential (178). The molecular weight of the pig liver enzyme has been estimated to be 270,000 daltons by sedimentation equilibrium (179).

Contrary to its wide occurrence in vertebrates, betaine:homocysteine S-methyltransferase has been reported to be present in only one invertebrate, the pond mussel, Anodona cygnea (177). Filarial worms are no exception to other invertebrates; methionine biosynthesis by this pathway could not be detected in extracts of these parasites (Table 3).

The finding that filariae are unable to synthesise methionine is consistent with the report of Tkachuck et al. (180) that B12-dependent methyltransferase activity has not yet been detected in any parasitic helminth whereas the enzyme is found in the free-living nematode Caenorhabditis briggsae (181). Parasitic nematodes therefore probably derive their methionine from an external source (182) unlike mammals, where even though this amino acid is considered "essential", they still have the capacity to synthesise a limited amount of it by the two pathways. On the other hand, the ability to synthesise methionine has been claimed to occur in certain protozoan parasites. Langer et al. (183) observed that in P.berghei, de novo synthesis contributed 20% of the total amount of methionine. This minimal level of biosynthesis has prompted Bueding to question whether activation of proteolytic release of methionine by homocysteine might not explain the data of Langer et al. (183). Although methionine

formation is found in Crithidia fasciculata, it is by a novel pathway. This kinetoplastic flagellate possesses a folate-dependent mechanism for producing α-keto-hydroxybutyrate from phosphoenolpyruvate and the β-carbon of serine (184). In the presence of organic sulphur, methionine is formed.

The inability of adult filariae to synthesise methionine de novo may be an adaptive feature, in which case the deficiency might be expected to exist in all the developmental stages. This is suggested by the pattern of change in folate-related enzymes in filaria-infected mosquitoes. Jaffe et al. (122,123,125-127) observed an elevation in the activities of dihydrofolate reductase, serine hydroxymethyltransferase, CH_2THF reductase and methionine synthetase whereas the level of 10-formylTHF synthetase correspondingly declined. It was suggested that the developing filarial larvae might be depleting the mosquitoes of their methionine or N^5-methylTHF stores or both. This picture may, however, be different in helminth parasites with free-living stages. In these helminths it is possible that some of the enzymes apparently absent or inactive in the parasitic adults may in fact exist and function in the free-living developmental stages.

(b) ATP: Methionine S-Adenosyltransferase

Although they differ from mammals by being unable to synthesise methionine, filarial worms are similar to mammals in that they have the enzymatic machinery to metabolize this amino acid as indicated by Tables 2 and 3. Filariae also resemble Plasmodium knowlesi in this respect. P.knowlesi was demonstrated in vitro to convert (^{35}S)methionine into (^{35}S)cystine (185). The levels of the enzymes catalyzing the transformation of methionine to homocysteine and adenosine in filariae

are about two to three orders of magnitude less than the corresponding ones in the rat liver; the levels of the transsulphuration enzymes are between 1 to 10 times lower in activity than the analogous mammalian enzymes (186,187).

Activation of methionine appears important in the further metabolism of this amino acid. In a classical contribution, Cantoni (188) identified the active form of methionine as being AdoMet; adenosylation of L-methionine at the expense of ATP, catalyzed by AdoMet synthetase appears to be the sole pathway for the formation of AdoMet (189). AdoMet synthetase is ubiquitous in nature (190,191). The fragmentary studies of the tissue distribution of AdoMet synthetase in mammals suggest that the enzyme is most active in the liver, with females exhibiting a higher activity than males (192-194).

Owing to the instability of AdoMet in alkaline media, the activity of the enzyme has usually been determined around neutral pH (195-196). But where the ATP-Mg^{++} ratio has been optimized, an activity peak at pH 9.0 has been reported (197). Below pH 7.8, Mn^{++} was found to be as effective as Mg^{++}. In addition to divalent cations, AdoMet synthetase also requires monovalent cations such as K^{+}, Rb^{+}, or NH_4^{+} for maximal activity (197).

The limited information available on the nucleotide specificity of AdoMet synthetase suggests that only ATP (189) and to a very minor extent UTP in mouse liver (199) are active. Data on the specificity of methionine show that only few modifications would be compatible with enzyme activity (200). The enzyme requires a carboxyl group (or short ester) and an amino group (or short acyl... derivative) attached to a

carbon atom bearing a hydrogen atom and linked by a two carbon bridge to an ether sulphur (or selenium) atom bearing a short alkyl substituent (either methyl or ethyl). In the course of the formation of AdoMet there has been observed product-activated tripolyphosphatase activity (189,196,134,201-203) which has led to the suggestion that an enzyme-bound tripolyphosphate might be an intermediate in the reaction mechanism.

Chiang and Cantoni (132) recently purified bakers' yeast AdoMet synthetase to homogeneity; two forms of the enzyme were demonstrated. The apparent molecular weight for both forms of the enzyme as determined by Sephadex G-150 column chromatography was 110,000 daltons, in agreement with a value of 100,000 daltons obtained by Green (197) using analytical ultracentrifugation. Molecular weights of 150,000 and 45,000 daltons have also been reported (196,204).

As indicated earlier, the level of ATP: methionine S-adenosyltransferase in filariae is very low. This might possibly account for the unsuccessful attempts at characterizing this enzyme. The low activity might in turn be attributed to the state in which some of the worms were received. Some of the worms were received surrounded by water, the ice having thawed.

(c) Metabolic Functions of S-Adenosylmethionine

Once synthesised, AdoMet is utilized in various reactions. It participates in most biological methylations (Table 11) by donating its methyl group to acceptor molecules. Not only is it a methyl donor, the relatively high energy of AdoMet provides the energy for the largely exergonic transmethylation reactions (200).

TABLE 11

Some Examples of Transmethylation Reactions Involving AdoMet

Acceptor Molecules	Product
N-Acetyl-5-hydroxytryptamine	Melatonin
Carnosine	Anserine
Epinepherine	Metanephrine
Guanidinoacetate	Creatine
Histamine	N-Methylhistamine
Nicotinamide	N'-Methylnicotinamide
Norepinephrine	Epinephrine
Phosphatidylethanolamine	Phosphatidylcholine
Lysyl residues of proteins	N-Methylated lysyl residues
Carboxyl groups of proteins	Methylesters
Purines	Methylated purines

Lipids constitute an important chemical pool for the metabolism of methyl groups in animals. Filariae appear to be no exception; twenty-one percent of radioactivity of (CH_3-^{14}C)methionine were found in the lipid fraction. The level of radioactivity incorporated into the total lipids of filariae compares with that obtained in other systems. Studies have shown that after a one hour incubation of Trypanosoma equiperdum in (CH_3-^{14}C)methionine, 8% of the radioactivity were recovered from the total lipid fraction (205), whilst 27-34% of the label were incorporated into the total lipid fraction of two strains of Aerobacter aerogenes (206).

Almost all the radioactivity in the lipid fraction was found in the phospholipid component. The recovery of radiolabelled phospholipids clearly shows that the unlabelled phospholipids isolated from female D.immitis (Plate 1) and other parasitic nematodes (102,199) do not originate solely from the host but that these parasites also possess a biosynthetic capacity for these compounds. The results further reveal the existence in filariae and probably other parasites of a transfer of methyl groups from methionine to phosphatidylethanolamine in a similar way to the process described in vertebrates (207) and invertebrates (208). This is a stepwise methylation reaction catalyzed by two microsomal methyltransferases (209-211). Methyltransferase I (a Mg^{++} requiring enzyme) has a high affinity for AdoMet (Km of mammalian enzyme 1.4 uM) and catalyzes the formation of phosphatidylmonomethylethanolamine (PME). Methyltransferase II mediates the conversion of PME through phosphatidyldimethylethanolamine (PDE) to phosphatidylcholine. It has a Km for AdoMet of 100 uM. This pathway is estimated to contribute 20-40% of the total lecithin pool in mammals (212), cytidylyldiphosphate-choline incorporation into a, p-diacylglycerate (213) accounting for the remainder.

In addition to detecting radioactivity in lysolecithin and lecithin, counts were also observed in sphingomyelin. But the formation of the latter needs clarification. The terminal phosphocholine moiety of sphingomyelin could be derived from phosphatidylcholine by the action of phospholipase C or D or a nonspecific phosphatase. The labelled phosphocholine or choline released could either interact with unlabelled sphingomyelin in an exchange reaction or complex with ceramide, the parent compound of sphingolipids, in an energy dependent reaction.

In addition to the biosynthesis of phosphatidylcholine, trypanosomids also possess the ability to incorporate methionine into their sterols. Meyer and Holz (214) found that Crithidia fasciculata incorporated (CH_3-^{14}C)methionine into ergosterol. The bloodstream and culture form of Trypanosoma lewisi and culture form of T.rhodesiense also synthesized some unidentified sterols from acetate and methionine (111).

The formation of fatty acid methylesters which has been reported in several biological systems incubated with (CH_3-^{14}C)methionine (215) could not be followed in the present study because of the inavailability of the appropriate markers. Although the silicic acid-celite column would resolve these substances from the phospholipids, inefficient separation could explain the radioactivity at the solvent front since fatty acid methylesters migrate ahead of the phospholipids in the development solvent employed in this experiment. But there was not enough time to try other fractionation systems to completely separate the phospholipids and the methylesters. However, it has been observed from other studies that the presence of the latter substances could be artifactual as extracts in methanol could induce either enzymatic or chemical transesterification reactions (215).

The amount of radioactivity retrieved from the filarial protein is similar to the level reported for A.aerogenes (206) and T.equiperdum (205),

The isolation of methylated lysine, histidine and arginine from filariae is a further manifestation of the ubiquity of protein methylation (144). Several amino acid residues are known to be methylated usually at the nucleophilic atoms by specific methylases. N-methylation at lysyl, argininyl and histidyl residues and O-methylation at free carboxyl groups are catalyzed by specific AdoMet-dependent methyltransferases. These covalent modifications of amino acid residues have been implicated among the posttranslational mechanisms for controlling protein function (144).

Ɛ-N-Methylated lysine residues are known to occur in a wide variety of proteins from many sources (216). Among these cytochrome c's from fungi, plants and protozoans have been found to possess one or more of this amino acid (216). In contrast, none of the cytochrome c's of either vertebrates or invertebrates possess Ɛ-N-methylated lysine. The Ɛ-N-trimethyllysine of trypanosome cytochromes makes the pigments of the parasites less basic than the vertebrate cytochromes (111). Methylation of cytochrome C of yeast or Neurospora crassa has been shown to facilitate the binding of the cytochrome with mitochrondria, and subsequently play an important role in the process of electron transport (144). Although actin and myosin also contain significant amounts of various methylated basic amino acids the biochemical significance of protein methylation in muscle tissues is unknown at present (217). Histones are also heavily methylated and the results suggest that this phenomenon is one of the many factors which lead to repression of DNA prior to mitosis (217). While methylated amino acids have been demonstrated in filariae, the proteins are yet to be identified.

It is apparent from the results that filariae are unable to synthesize methylated bases (Table 10). It is, however, possible that the turnover of the enzymes mediating these reactions in filarial worms is so low that a much longer incubation

period should have been used. There are also doubts about the presence of methylated bases in other parasites. A study of nucleic acids of Plasmodium berghei did not reveal the presence of any methylated bases but it was observed that the extraction procedure was not appropriate to resolve these compounds (183). Attempts by Aronson and Jaffe (205) to demonstrate the presence of 5-methylcytosine in T.equiperdum were also futile. Incorporation of radioactivity from [CH_3-^{14}C]methionine into the nucleic acid fraction can probably be regarded as artifactual. The presence of 5-methyl cytosine, however, has been demonstrated in other eukaryotes. It is estimated that between 2% and 8% of the cytosines of species from the Coelentera, Mollusca, Annelida, Echinodermata and Chordata are methylated (218).

The observation that the addition of potassium cyanide led to an increase in the activity of filarial AdoMet synthetase is an indirect indication that extracts of these parasites exhibit AdoMet decarboxylase property whose catalytic activity produces decarboxylated AdoMet, the primary role being for the synthesis of polyamine (202,219-222). Previous studies have shown that AdoMet decarboxylase activity is abolished by carbonyl reagents including cyanide, phenylhydrazine and semicarbazide (223). Polyamine formation is common in prokaryotes and eukaryotes. Recently bloodstream forms of Trypanosoma brucei were reported to produce polyamines (224). It is widely believed that polyamines have important regulatory roles in mammalian cell physiology, but these are at present uncertain.

Other metabolites of AdoMet whose presence in filariae was not investigated are briefly considered below because of their physiological importance. Bacteria, baker's yeast and mammals metabolize AdoMet to 5'-methylthioadenosine (MTA) but the microorganisms also produce amino-γ-butyro-lactone (200,144). In Aerobacter aerogenes but not in yeast, 2-amino-3-butenoic acid appears to be the initial product in the formation of butyrolactone (200). Further metabolism of MTA gives 5'-methylthioribose and adenine in bacteria and 5'methylthioribose-1-phosphate in mammals (144). Adenine is the second product in both systems. In both prokaryotes and eukaryotes there are speculations that the methylated thioribose may eventually produce methionine (144). MTA is considered a potent regulator of enzyme systems and it has been demonstrated to inhibit proliferating human lymphocytes (144).

The demethylated product of AdoMet, AdoHcy, also goes through multiple routes of transformation. In bacteria, but possibly not in yeast and mammals, AdoHcy is cleaved by a nucleosidase to S-ribosylhomocysteine and adenine (200) the former product undergoing further metabolism to give homocysteine and ribose (225). There is evidence that the same bacterial nucleosidase catalyzes the formation of 5'-methylthioribose from AdoMet. In mammalian systems, however, the action of L-amino acid oxidase on AdoHcy results in the formation of S-adenosyl-γ-thio-α-ketobutyrate (200). Non-specific adenosine deaminase of Aspergillus oryzae also deaminates AdoHcy as well as MTA and in the case of the former to S-inosylhomocysteine (200). Although none of these transformed products of AdoHcy were investigated in filariae, it is possible that these parasites may have such mechanisms owing to the widespread nature of these products of AdoMet.

By far the most common pathway of AdoHcy metabolism in eukaryotes is by way of AdoHcy hydrolase (22°) which has been identified in filariae (Tables 2 and 3). AdoHcy hydrolase has been extensively studied because it may be crucial in the regulation of tissue levels of AdoHcy (226-228). The enzyme has been found in all rat tissues examined, with especially high levels in the liver and pancreas (144,186). Mammalian AdoHcy hydrolase appears to have an optimal pH around 6.5 (144). Apparently the same enzyme catalyzes both the forward and reverse reactions (186,229). The condensing enzyme seems to exhibit an absolute requirement for homocysteine whereas some structural modifications of the nucleoside can be tolerated (229,230). Calf liver AdoHcy hydrolase was recently purified to homogeneity (231). Molecular weight determination of the native enzyme by gradient gel electrophoresis in the presence of sodium dodecylsulphate has suggested that AdoHcy hydrolase might be a tetramer.

It is apparent from spectroscopic studies that calf liver AdoHcy hydrolase contains a tightly bound NAD^+ (231,232) prosthetic group; this NAD^+ has been implicated in the mechanism of action of the enzyme. Addition of adenosine to AdoHcy hydrolase caused an increase in the absorbance at 327 nm which was attributed to NADH formation.

Although the equilibrium of the reaction favours the formation of AdoHcy (229), the enzymatic removal of homocysteine and adenosine (end product inhibitors of the hydrolase) (200,229) should ensure a unidirectional transformation of methionine to cysteine. Filariae can convert adenosine to purine nucleotide by way of adenosine kinase or after its deamination to form inosine and eventually hypoxanthine, by way of hypoxanthine:guanine

phosphoribosyltransferase (267). The properties of these auxilliary enzymes have also been investigated in mammals and microbial systems (233,234).

Homocysteine is utilized in the following reactions:

a. Synthesis of methionine by the vitamin B12-independent and dependent pathways (156,160,179,187,200).

b. AdoMet-dependent methylation of homocysteine to methionine (200).

c. Condensation with serine to form cystathionine a reaction irreversible in mammals but reversible in higher fungi e.g. Neurospora crassa (200, 235,236).

The absence of the first reaction in filariae suggests that filarial homocysteine can participate in only reactions (b) and (c). Attempts to show the formation of homocysteine from cysteine (an irreversible pathway, in at least two species of bacteria (163))met with no success in the filariae. In mammals and microbes the kinetic constants of the components of methionine synthesis suggest that low levels of tissue homocysteine should favour the activity of N^5-methylTHF:homocysteine S-methyltransferase (144). This presupposes that AdoMet:homocysteine methyltransferase will be at a kinetic advantage owing to its high affinity for homocysteine. On the other hand elevation of homocysteine level would commit this thiol compound to transsulphuration just like the situation in mammalian systems. Of course this depends on whether the filarial synthase shows a similar kinetic constant; at the time of writing, insufficient quantities of this filarial enzyme were available to permit such kinetic analyses.

(d) AdoMet:Homocysteine S-Methyltransferase

Methionine formation by the AdoMet-mediated direct remethylation of homocysteine has been observed in several biological systems.

Law et al. (237) have demonstrated that AdoMet:homocysteine methyltransferase is present in cell-free extracts from 1-hr Musca domestica embryos. Activity of the transmethylase has also been detected in Candida utilis (238), a number of strains of A.aerogenes (206), S.cerevisiae, E.coli strain K-12 and mammals (130,239,240) and now in filariae.

The level of the filarial methyltransferase is comparable to that of the corresponding enzyme in mammalian liver, which is the major site of methionine metabolism (144). The filarial transmethylase was partially purified with a purification factor of 5 and a yield of 94% (Table 4). The properties of the filarial enzyme are similar to the isofunctional microbial transmethylase with only minor variations. The microbial enzyme has been shown to be specific for the methyl donor, S-adenosyl-L-methionine (241). The dextro-rotatory methyl sulphonium compound and other methyl donors which are active in the thetin-homocysteine methylpherase system of liver were ineffective (175,176). Although it was not demonstrated in the filarial system, it is known in prokaryotes and other eukaryotes that S-methyl-L-methionine can substitute for AdoMet. Moreover, the same methylase appears to act on both methyl donors as depicted by the constant ratio of activity during fractionation of the enzyme from S.cerevisiae (240). Since AdoMet is the only substrate which has been identified in animal cells, (S-methylmethionine, apparently occurs widely in plants (242)), and it has a Km of 8 uM for the filarial transmethylase it is suggested that this compound may be the natural substrate for this filarial enzyme (Table 11). Two Michaelis constants for the baker's yeast transmethylase for AdoMet have been reported:

8.6×10^{-4}M (241) and 2.2×10^{-3}M (243). The apparent variation in the baker yeast transmethylase Km may be attributable to different batches of yeast which is not an uncommon phenomenon in biological studies. The Km for M.domestica embryo transmethylase is 2.8×10^{-3}M (237). The higher affinity exhibited by the filarial enzyme is comparable to that shown by mammalian AdoMet-utilizing methylases (144).

In contrast with the stereospecificity of the methyl donor, AdoMet:homocysteine methyltransferase is not stereospecific for the thiol acceptor. Both stereoisomers had demonstrable activity for the baker's yeast enzyme (241). But extracts of A.aerogenes could utilize only the levorotatory homocysteine (241). While this could not be investigated in the filarial system, it was shown that deletion of L-homocysteine decreased the activity drastically (Table 6). However, Shapiro et al. (241) have suggested that homocysteine racemase activity in the purified yeast extract might be responsible for the action on D-homocysteine. Likewise a lactonase activity has been assumed to explain the effect of the methylase on homocysteine thiolactone (241). The Km of homocysteine for the transmethylases from S.cerevisiae and filariae are comparable, being 2.3 mM (243) and 0.9 mM (Table 9) respectively. A Km of 0.32 mM has also been reported for the yeast methylase by the same group of investigators (241).

In addition to functioning as a methyl acceptor, the prevailing notion is that homocysteine may also be acting as a reducing agent. Shapiro et al. (241,243) observed that a 15 min preincubation of S.cerevisiae extract with homocysteine was necessary in order to obtain linearity with time. In spite of this preincubation, the rate of reaction

as a function of homocysteine concentration was not proportional. Subsequent experiments with various reducing agents confirmed the reducing properties of homocysteine. Mercaptoethanol which was the most effective appeared to reduce the yeast enzyme; cysteine, thiodiglycol and 2,3-dimercaptopropanol also produced marked increase in the rate of reaction. In the case of the filarial transmethylase it was noticed in the course of partial purification that the mercaptoethanol in the extraction buffer was responsible for the observed thermal activation following preincubation of the enzyme at 50°C for 5 min. It was considered that mercaptoethanol was either protecting the filarial enzyme against heat denaturation or acting as activator or both. This prompted a study of the effect of thiol compounds on the activity of this enzyme. Among a variety investigated, cysteine was a significantly better activator of the enzyme than either dithiothreitol, reduced glutathionine or 2-mercaptoethanol. The inclusion of reduced glutathione in the incubation mixture for measuring the mammalian methyltransferase activity (130) suggests that the other methyltransferases may also require thiols for the expression of their optimal activity.

In all these cases it did not appear that the reducing agents were also replacing homocysteine as methyl donor. A mammalian thiolmethyltransferase that catalyses the methylation of a variety of non-physiological sulphydryl compounds such as mercaptoethanol has been reported (245-246). In contrast with the yeast and filarial transmethylases which are cytosolic, the above mammalian methyltransferase is microsomal and independent of divalent metal ions for the expression of its catalytic activity. The involvement of metal ions in the catalysis of Adomet:-homocysteine methyltransferase is variable. The bacterial and yeast

transmethylases were activated by Zn^{++} and Cd^{++}; but Mn^{++}, Ni^{++} and Co^{++} were without effect (241). In addition to stimulation by transition divalent metal ions, alkaline earth metal ions, Mg^{++} and Ca^{++}, also activated the filarial transmethylase (Table 7). By contrast the corresponding mammalian enzyme does not appear to require metal ions for activity, since neither EDTA nor 1,10-ortho-phenanthroline affected its activity (247); both chelators, however, inhibited the filarial and microbial transmethylases (Table 8 and 247). Even though participation of divalent metal ions in the methylase activity of filariae and microbes has been demonstrated, in none of these enzymes has an absolute metallic requirement been shown. This seems to confirm the observation of Malmstrom and Rosenberg (248) that there is no absolute specificity in metal ion activation of enzymes; since in all cases investigated more than one metal ion has pronounced effect on the catalytic activity of the protein. However, there are usually distinct differences in the efficiency with which different metallic ions act as activators for given enzyme as has been shown for these transmethylases.

The optimum pH exhibited by filarial transmethylase (Fig. 3) is similar to that of S.cerevisiae (240); in both cases around pH 8.0. However, the rate of the enzyme reaction has usually been measured at pH values lower than the optimum so as to avoid the chemical decomposition of AdoMet which takes place in alkaline media (195-198).

The optimum temperature of filarial AdoMet:homocysteine methyltransferase of 50°C (Fig. 5) appears to be unusually high; this may be related to the presence of mercaptoethanol in the reaction mixture as pointed out previously in this discussion. Studies on the effect of

temperature on the analogous enzyme in jack bean meal revealed that the optimum activity occurred at 60°C (242).

With the exception of glycine methyltransferase which is weakly inhibited by AdoHcy, all AdoMet-utilizing transmethylases are potently inhibited by the thiol ether product (144,249). The effect of AdoHcy on AdoMet:homocysteine methyltransferase is uncertain. The microbial methylase was reported to be inhibited by AdoHcy (241). However, Ferro and Spence (250) observed that the level of the enzyme in cells of S.cerevisiae grown in 0.1 mM AdoHcy was unaffected. It could be that the effective concentration of AdoHcy was not achieved because of its metabolism in the cells or due to the lack of a transport mechanism of AdoHcy in the yeast. The filarial and M.domestica transmethylases were refractory to inhibition (see result section, 237). Although no information exists on the intracellular levels of AdoMet and AdoHcy in filariae, the value of 0.045-0.060 umol/g wet weight in mammalian liver (237) suggests that in vivo, the filarial transmethylase still would be uninhibited. On the contrary AdoMet: homocysteine methyltransferase from filariae was apparently susceptible to inhibition by methionine. It was observed during studies on the relationship between enzyme concentration and rate of reaction that a negative slope resulted if the level of AdoMet was 2 mM. It was lucidly demonstrated in S.cerevisiae that there was 50%

inhibition of the rate of reaction with as low as 0.4 umole of added methionine (241). The rate of methionine formation was linear only if its concentration was less than 0.5 umole/ml of reaction mixture (240).

Although a long period has elapsed since the introduction of DEC and suramin in the chemotherapy of filariasis, exactly how they exert their antifilarial action is still uncertain. The strong sensitivity of some folate-related enzymes to the inhibitory action of these drugs has been observed. DEC has been demonstrated to strongly inhibit filarial serine hydroxymethyltransferases, CH_2THF dehydrogenases, NADP-independent 10-formylTHF dehydrogenase as well as B.pahangi CH_2THF reductase (117) whereas it had marginal or no activity against the corresponding mosquito enzymes (122,125). Jaffe and associates (57) also found that filarial dihydrofolate reductase and NADP-dependent 10-formylTHF dehydrogenase were sensitive to inhibition by suramin; however the isofunctional mammalian and mosquito dehydrogenases NADP-dependent (58) and mosquito CH_2THF reductase, 10-formylTHF synthetase (122,123), and thymidylate synthetase (124) were also strongly inhibited by suramin. The activities of trypanosomal α-glycerophosphate oxidase, dihydrofolate reductase, thymidylate synthetase and dihydroorotate hydroxylase were also blocked by suramin (251,252). In spite of the interference with folate metabolism and hence also folate-dependent reactions in filariae caused by these filaricides these effects may not represent their only mode of action. Suramin has been demonstrated to be a potent inhibitor of a variety of other enzymes including those involved in carbohydrate metabolism and DNA replication and transcription (19,253). As the plasma concentration of these filaricides is lower than the level utilized in this study

(19,20) it seems unlikely that *in vivo* suramin and DEC might be exerting their antifilarial action, even in part, by inhibiting AdoMet:homocysteine methyltransferase.

As earlier indicated, although prokaryotic and eukaryotic cells were found able to form methionine by the AdoMet-mediated direct remethylation of homocysteine, the physiological significance of this is obscure. It has been suggested that the high turnover in the methionine pool of the cell would make AdoMet:homocysteine methyltransferase a fail-safe mechanism for the synthesis of methionine should the level of this amino acid decline below a critical point (130,200). It is thought that this will ensure the functionality of AdoMet synthetase, which has a high Km for methionine, the tissue level of which is low. It has been demonstrated that when *S.cerevisiae* were grown under conditions where the intracellular concentrations of AdoMet were elevated there were repression of three methionine metabolic enzymes: homocysteine synthetase, ATP-sulphurylase, and AdoMet synthetase with a concomitant induction of AdoMet:homocysteine methyltransferase (250). It was therefore suggested that the enzyme might regulate AdoMet and methionine balance and synthesis. It was further considered that this induction-repression phenomenon would prevent the superfluous, though inevitable cyclization of methionine which would occur were AdoMet synthetase and AdoMet:homocysteine methyltransferase to operate simultaneously.

Law and colleagues (237) have proposed that AdoMet:homocysteine methyltransferase may be functioning to regulate the activity of the tRNA methyltransferases. They noticed an inverse relationship between the activities of these transmethylases during embryogenesis of *M.domestica*

(254); the activity of AdoMet:homocysteine methyltransferase declined with progression of embryonic development. A similar observation was made by Kuchino et al. (255) in hepatoma cells in which elevated levels of tRNA methyltransferase activity were noticed with a total absence of AdoMet:homocysteine methyltransferase. Inverse relationships have also been established between glycine-N-methyltransferases and tRNA methyltransferases in neoplasms, fetal rabbits and aging rodents (249,254,256).

C. AdoMet-Mediated Methyltransferases as Potential Targets in the Chemotherapy of Parasites: As indicated elsewhere in this text, transmethylation reactions utilizing AdoMet are sensitive to inhibition by AdoHcy. It has been shown that the sensitivity of these methylases is greatly influenced by changes in AdoMet/AdoHcy ratio (144). The activity of AdoHcy hydrolase is greater than that of AdoMet synthetase in all cells examined so far and the concentration of AdoMet is usually 3 to 4 times larger than that of AdoHcy. On the basis of these findings emphases have been directed to designing analogues that would interfere with AdoMet/AdoHcy ratio ultimately leading to the control of the utilization of AdoMet.

One approach has been to alter the activity of AdoMet synthetase. Inhibition of this enzyme would lower the concentration of AdoMet thereby inhibiting all AdoMet-dependent reactions. Of the several analogues of methionine that were studied, 1-aminocyclopentane-1-carboxylic acid (cycloleucine) and 2-amino-4-hexynoic acid proved potent (203,257,258).

AdoMet analogues have also been evaluated for their activity against AdoMet-dependent methyltransferases. Sinefungin and A9145C isolated from Streptomyces griseolus were the outstanding members of this group of inhibitors (146).

But the approach which has attracted more considerable attention is reduction of the activity of AdoHcy hydrolase which will result in an increase in the concentration of AdoHcy. An analogue which is a powerful inhibitor of AdoHcy hydrolase is 3-deazaadenosine and its administration has been shown to produce pronounced biological effects such as decrease in creatine and phosphatidylcholine in rat liver (259), antiviral activity against Rous sarcoma virus in chick embryo fibroblasts and Gross murine leukemia virus in mouse embryo cells (260). 3-Deazaadenosine has also been shown to produce antimalarial activity against Plasmodium falciparum in culture (259).

Recent findings indicate that AdoMet-dependent methylations might regulate important physiological activities whose study in filariae and related parasites may likely furnish answers to some of the baffling biochemical and physiological peculiarities which characterize the parasitic adaptation. Biological methylation appears to be important in parasites in the area of phospholipid synthesis and protein modification.

Phospholipids are a major component of biomembranes and provide the fluid matrix for movement of other membrane components (144). The cuticle of filariae, as of other parasites has high level of lipid of which phospholipids are among the dominant ones (113); Ascaris lumbricoides cuticle, for instance, is composed of 38% phospholipids (107). The heavy demand of filariae for phospholipids is indicated by the absence of betaine:homocysteine methyltransferase (Table 5) which has been suggested by Finkelstein et al. (261) to metabolize excess homocysteine and betaine. It has been observed that parasites readily alter their outer coat to make immunological recognition less possible. Extensive

antigenic variation has been noticed in trypanosomes; different clones of T.brucei have been found to contain diverse glycoproteins (262). In schistosomes, electron microscopy suggests that the vitiation of host response depends on continuous membrane activity at the schistosome integument. It is likely that these membranal activities are associated with a rapid turnover of the parasites phospholipids. In addition there should be a close parallelism between these cuticular changes and phosphatidylcholine - lysophosphatidylcholine interconversion or phospholipases A1 and A2 activities. Lysophosphatidylcholine released by the action of these lipases is lately being regarded as a natural fusigen fusing the lipid bilayers together. It is possible then that perturbation of parasites AdoMet/AdoHcy ratio coupled with inhibition of phospholipases A1 and A2 might help interrupt the periodic transformation of the surface coat.

AdoMet is further involved in the rudimentary nervous system of parasites. Acetylcholine is an inhibitory neurotransmitter in S.mansoni (111). Its presence in filariae has been inferred from the detection of cholinesterase activity in D.viteae (44). Serotonin acts as a stimulatory transmitter in addition to its regulatory role in the carbohydrate metabolism of trematodes (111). Moreover, sphingomyelin which is a constituent of the nervous tissue has been reported to be present in some parasites including filariae (102, Fig.12). These substances, however, are partly either produced or degraded through methylation, for instance depolarization and glucose depletion may occur in flatworms if the AdoMet-mediated catabolism of serotonin is blocked.

Like phospholipids, protein methylation is also of vital importance to the cell, although little is known about this process in parasites. Modification of protein by methylation is associated with many diverse phenomena such as bacterial and leucocyte chemotaxis, and peptide hormone degradation and neurosecretion (144).

The enzymatic methyl esterification of the membrane polypeptide is an area worth investigating. Such a reaction will result in charge/potential alteration of the membrane and may influence charge-related membrane function, shape and structure of the cuticle. This would affect the transcuticular uptake of nutrient which Chen and Howells (115) have suggested might be relevant in filariae. Interestingly, the only major methylated polypeptide in rabbit erythrocyte membranes has also been implicated as a component of the glucose transport system of the erythrocyte (144) which like filariae and other helminth parasites depends principally on phosphorylative glycolysis for energy. Equally important in filarial worms is methylation of protein lysyl and arginyl residues. Methylation of lysyl groups is mediated by protein methylase III whose activity is elevated in heavily proliferating cells (145). As female macrofilariae have a high reproductive capacity they should have a high activity of this enzyme. Microfilariae and other developmental stages of parasites should also possess considerable amounts of the enzyme. Selective inhibition of this enzyme might be detrimental to the filarial worms.

Chemotactic responses of organisms to harmful and beneficial environmental substances have been linked to the completion of the life cycle of helminth parasites especially those with free living stages (263). Amino acids have been implicated as the stimuli attracting miracidia of schistosomes to their molluscan vectors (259). This has

led to the suggestion of the inclusion of amino acid analogues into larvicides to decoy the miracidia. Furthermore, proline and glutamate have been identified as chemical attractants for Biomphalaria glabrata, the snail host of S.mansoni (263). It is thus possible to use these amino acids as baits to trap the snails. Chemotaxis, however, is an AdoMet-dependent transmethylation process (144). It has been demonstrated in both prokaryotes and eukaryotes that in the presence of an attractant there is an increase in the methylation of so-called Methyl-accepting Chemotaxis Protein (MCP) located in the cytoplasmic membrane whereas the converse takes place with the repellent (144). These reactions precede the movement of the organism in response to the stimulus. The observation that methylation deficient mutant strains or cells depleted of AdoMet are defective in chemotaxis (144) means that interfering with the parasitic helminths methylation apparatuses might yield effective results even though the environmental stimuli may be available.

The stability of mRNA depends on its methylated cap structure, $m^7G^{5'}ppp^{5'}N$, at the 5'-terminus (144). Capped mRNAs facilitate initiation of protein synthesis by promoting mRNA binding to ribosomes. AdoMet-utilizing tRNA methylases are among tRNA modifying enzymes that confer species differences to the highly conservative secondary structure of tRNAs (145).

It seems inconceivable that with only four major nucleotide nitrogenous bases giving a triplet code of 64 codons there should evolve wide physiological and morphological variations in nature. DNA methylation which occurs prior to and immediately after the S-phase is thought to explain this paradox. It has been suggested that it would also protect the DNA against integration with a foreign DNA and the action of DNAases; as,

for example, bacterial restriction and modification enzymes which are mechanisms for the recognition and degradation of invading foreign DNA.

Although no radioactivity was detected in the nucleic acid fraction of *D.immitis* (Table 10) during the six-hour period of incubation in (CH_3-^{14}C)methionine, the current notion that modification of nucleic acid by AdoMet-dependent methyltransferases is common in prokaryotes and eukaryotes (146) makes this area in filariae worth pursuing because if there exists a species-specific DNA methylation pattern, which seems plausible, it might be an exploitable site for antiparasitic chemotherapy.

There is no doubt from the foregoing that AdoMet-mediated methylation reactions may offer new insights into the physiology and biochemistry of filariae and related parasites; but one wonders whether with the versatility and significance of these reactions, usage of methyltransferase inhibitors would provide any selectivity. For instance, inhibition of AdoMet synthetase and AdoHcy hydrolase will result in a general inhibitory effect on all AdoMet-dependent methyltransferases. Nevertheless, the situation with dihydrofolate reductase inhibitors is a perfect analogy. Although dihydrofolate reductase is ubiquitous, a selective effect on rapidly proliferating cells by these antifolates forms the basis of their action (264). Therefore the knowledge gained with the use of folic acid analogues can be extended to AdoMet-dependent methylation reactions. For example, as the ability of some parasites to evade the host immune systems implies a high membrane turnover, and with phospholipids being one of the major constituents of membranes, it is envisaged that parasites will be more prone to the adverse effect of phospholipid methylation inhibitors. As with dihydrofolate reductases the following factors should be investigated, namely: (1) relative rates and transport mechanisms of methylases inhibitors between host and,

parasite, (2) degree of binding and (3) relative rates of metabolism of inhibitors in host and parasite, and (4) existence of isoenzymes with different binding characteristics.

Since methylation reactions in living systems are numerous, the prospect of achieving selectivity on the surface appears remote as a whole array of important reactions may be blocked. It has, however, been reported that in humans about 89% of the net flow of methionine through AdoMet is involved in the formation of creatine (265,266); guanidoacetate methyltransferase which catalyzes this reaction has not been detected in any invertebrate (266). Although this difference is important, it is still not enough to afford selectivity. Being an AdoMet-dependent enzyme, guanidoacetate methyltransferase should be affected by AdoMet-mediated methyltransferase inhibitors directed against enzymes of the infective agent. Thus exploitation of the absence of guanidoacetate methyltransferase in parasites seems impossible at this stage. However, kinetic differences between parasite and definitive host AdoMet-dependent methyltransferases could buttress the above basic variation between the two groups of organisms. As such kinetic parameters are non-existent, it is important that AdoMet-dependent methyltransferases in parasites and their primary hosts be actively investigated. These studies could yield some of the valuable kinetic differences that could be an asset chemotherapeutically. Hence, just as it has been achieved with dihydrofolic acid reductases, there is every chance that diligent study of biological methylation reactions and other aspects of methionine metabolism in parasites should provide exploitable leads to the synthesis of selective inhibitors.

In summary, adult filariae apparently cannot biosynthesise methionine, they, however, retain the catabolic capacity.

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