DESIGN OF POTENTIAL ANTIPROTOZOAL DAUNORUBICIN DERIVATIVES

by

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C O N T E N T S

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While registered as a candidate for the degree of Doctor of Philosophy at Leicester Polytechnic I have not been a registered candidate for another award of the CNAA, or of a University.

Mark Alan Hardman
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ABSTRACT

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Daunorubicin is an antitumour antibiotic which is highly active against the sleeping sickness parasite *Trypanosoma rhodesiense in vitro*, but which lacks *in vivo* activity. The object of this work was to modify daunorubicin so as to promote *in vivo* activity, and to study the mechanism by which daunorubicin is trypanocidal.

A series of daunorubicin analogues, and derivatives in which daunorubicin was linked to a macromolecular carrier (known as daunorubicin conjugates) were prepared, and tested against trypanosome infected mice. Only daunorubicin conjugates in which drug was linked to the carrier by glutaraldehyde, were active. Treatment with these conjugates increased the survival time of infected mice from three days to as long as eleven days, and temporarily cleared parasites from the bloodstream of infected animals. Conjugates with other types of linkage were inactive. A fluorescence assay method was developed to measure drug release from conjugates. Investigation revealed that glutaraldehyde linked conjugate released about 20% of bound drug over a 2-3 hour period when incubated in murine plasma. In contrast, inactive conjugates either released bound drug very rapidly, or were stable to drug release.

Daunorubicin is known to possess several potentially cytotoxic mechanisms of action, the most important being intercalation into the DNA double helix and stimulation of superoxide radical formation. In order to discover the contribution of these mechanisms to trypanocidal activity, the trypanocidal potency of a series of daunorubicin analogues was assessed *in vitro*. The ability of these analogues to intercalate into DNA and to stimulate lipid peroxidation and oxygen consumption was also assessed. The results were used to explore the relationship between these mechanisms and trypanocidal activity. These studies indicate that ability to bind to DNA is important in conferring trypanocidal activity on this group of antibiotics.

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CHAPTER 1 : INTRODUCTION

1.1 Parasitism as a major world health problem

Parasitic diseases are considered to be a major obstacle to social and economic progress throughout the Third World (Schultz, 1976), although they are no longer regarded as a major health problem among the developed nations. Parasitic diseases are very prevalent (Table 1), especially in tropical regions (Duke, 1978). The importance of parasitic diseases has been recognised by the World Health Organisation, and of the six diseases which it has selected for consideration in the Special Programme for Research and Training in Tropical Diseases, five are parasitic diseases (de Maar, 1979); (the sixth, leprosy, is a bacterial disease).

Table 1.1: Prevalence of human parasitic diseases

Disease	Number infected (10^{-6})	Reference
Ascariasis	1269	Peters 1978
Ancylostomiasis	932	Peters 1978
Schistosomiasis	271	Peters 1978
Onchocerciasis	20-40	Duke 1981
Filariasis	657	Peters 1978
Malaria	100	Brown et al 1976
Trypanosomiasis (Chagas disease)	13-14	Zeledon 1981
Trypanosomiasis (sleeping sickness)	0.01	Foulkes 1981
Leishmaniasis	0.4	World Health Organization 1979
Amoebiasis	400	Haworth 1975

Most parasites cause chronic diseases which, although rarely fatal, increase the infected individual's susceptibility to intercurrent infection and decrease his capacity for work (Peters, 1978). As well as suffering from their intrinsic effects, man is excluded from areas where transmission of pathogenic parasites is a high risk, and he is therefore unable to fully exploit the natural resources of the region. In areas of Africa worst affected by onchocerciasis(river blindness), where up to 15% of the inhabitants may be blind, the communities cease to be economically viable and the inhabitants are forced to desert the fertile, but infested, valleys (Duke, 1981).

The complicated life cycles of parasites provide many opportunities for controlling the diseases they cause. The use of chemicals to destroy the invertebrate intermediaries has assumed major importance in the last thirty years. However, environmental hazards associated with the use of chemicals have prompted the adoption of alternative, ecological methods of control, such as prevention of man-parasite contact and destruction of potential breeding places. (Rajagopalan and Shiffman, 1974). Whilst these methods are important, chemotherapeutic agents are essential for the maintenance of health, prevention of the spread of disease where infection by the parasite cannot be prevented, and by attacking the parasite, where it already exists in the body. High development costs and the uncertainty of an adequate return on investment have limited the introduction of new drugs for parasitic diseases, hence the search for new drugs needs to be intensified (de Maar, 1979).

Trypanosomiasis is a particularly important protozoal parasitic disease found in much of South America and tropical Africa. African

trypanosomiasis of domestic stock (nagana) is highly pathogenic to cattle, preventing the exploitation of much of the African savannah for cattle rearing (Wilson et al., 1963).

According to Duggan (1973), the human disease (sleeping sickness) if untreated "has the highest case fatality of any communicable disease except that of rabies". There have been no new drugs introduced for treatment of either the human or animal disease during the last twenty-five years, and many of the existing drugs are structurally related, hence cross resistance can sometimes occur (Williamson, 1976). There is an urgent need for fundamental research to develop new trypanocidal drugs which do not bear a close structural relationship to existing drugs in their mechanism of action (World Health Organisation, 1979).

1.2 Trypanosomiasis

Trypanosomes are parasitic protozoa of the genus *Trypanosoma* (order kinetoplastida) and are found in the blood and tissues of many animals throughout the world (Hoare 1970). Although most of the genus *Trypanosoma* do not produce disease in their hosts, some species cause serious disease in man and domestic animals. The genus *Trypanosoma* is sub-divided into the Stercoraria and the Salivaria (Hoare, 1964), these being distinguished by the manner (faecal or salivary), in which the parasites are transmitted by the arthropod vector. The most important stercorarian trypanosome is *Trypanosoma (Schizotrypanum) cruzi*, the causative organism of Chagas' disease in South and Central America (Ormerod, 1979). This parasite differs significantly from the salivarian trypanosomes in life cycle (Hoare, 1970), insect vectors (Zeledon and Rabinovich, 1981), and metabolism (Fairlamb, 1982; Gutteridge, 1981).

The salivarian trypanosomes cause the animal disease nagana in cattle and horses (*Trypanosoma (Trypanozoon) brucei brucei*, *Trypanosoma (Nannomonas) congolense* and *Trypanosoma (Duttonella) vivax*, surra in horses and camels (*Trypanosoma (Trypanozoon) evansi*) and the human disease sleeping sickness (*Trypanosoma (Trypanozoon) brucei gambiense* and *Trypanosoma (Trypanozoon) brucei rhodesiense*).

1.2.1 African Human Trypanosomiasis (Sleeping Sickness)

Sleeping sickness was first described in 1742 by John Atkins, a naval surgeon, but the cause was not established until 1902, when R.M. Forde and J.E. Dutton found trypanosoma in the blood of a steamboat captain on the River Gambia (Dutton, 1902). The parasite, which is morphologically identical to the animal trypanosome *T. brucei* (Bruce, 1895) was subsequently named *Trypanosoma brucei gambiense*. Following a massive outbreak of sleeping sickness in the Lake Victoria region, a commission, consisting of David Bruce, Count Aldo Castellani and David Nabarro was appointed by the Royal Society to investigate the disease, and by 1903 they had obtained evidence that the tsetse fly was the vector for sleeping sickness (Castellani, 1903). The disease had long been established in its primordial foci in the river basins of West Africa, where it was a chronic disease, well adapted to the local population (Duggan, 1970). By the end of the nineteenth century, European colonial powers had established regular traffic with inland areas of Africa, leading to the introduction of sleeping sickness into areas where it had previously been unknown. The disease flourished, and by 1906 half a million people in the Congo basin had died from the disease.

Owing to control and surveillance measures, sleeping sickness is now in a "state of controlled epidemicity" (de Raadt, 1976) with about 10,000 cases reported to the World Health Organisation each year (Foulkes, 1981). However, where surveillance is not maintained, the disease is quick to re-establish itself. This was demonstrated in Zaire between 1960 and 1961, during a period of civil war. The prevalance, which had been kept under 0.02% for several years, rose to epidemic proportions before systematic surveillance was restored (Scott, 1970).

Gambian sleeping sickness is found in the rain forest and moist savannah regions of tropical West Africa from the Sahara to the Namibian Desert (Ady, 1965). The disease is transmitted by 'riverine' tsetse flies of the *Glossina palpalis* group. Contact with man occurs at such sites as river crossing points and water holes where man and tsetse fly come in close contact (Scott, 1970). Man is the most important host for *Trypanosoma brucei gambiense*, and the disease is normally spread by a man-tsetse fly-man cycle (Scott, 1970). However Gibson et al (1978) demonstrated that pigs and dogs can also harbour the parasite enabling the disease to survive in uninhabited regions (Mehlitz et al., 1981).

By 1910 it became apparent that there are two kinds of sleeping sickness when Stephens and Fantham (1910) isolated a virulent strain of trypanosoma from a patient in Zambia. This trypanosoma was subsequently named *Trypanosoma brucei rhodesiense*. It is transmitted by flies of the *Glossina morsitans* group which inhabit the savannah plains of East Africa. Wild animals, especially bushbuck, are the usual hosts (Ashcroft, 1959), and man is at risk only when he encroaches upon tsetse areas either for farming or for hunting.

1.2.2 Clinical features of Sleeping Sickness

The onset and early stages of both Gambian and Rhodesian forms of the disease are similar. The first sign may be the development of a lesion around the site of the tsetse fly bite usually about 8-10 days after infection. This trypanosomal chancre is commonly seen in white, but rarely in black, patients (Apted, 1980). A high fever, starting about ten days after the original bite, and accompanied by headache and sometimes rigours and vomiting, is usual in both the Gambian and the Rhodesian diseases (Apted, 1980). Debility, changes in personality and bouts of fever are also characteristic; this phase of the disease may last for months in the Gambian form, but in the Rhodesian form it is much shorter. In the final stage of the disease, the parasites invade the central nervous system and may be detected in the cerebro-spinal fluid (Apted, 1970). This final stage is characterised by nervous tremors, fits and the daytime somnolence which gives the disease its name. In the final weeks, somnolence deepens into coma, and death soon follows, although often from a concurrent infection such as pneumonia. The natural duration of the Gambian disease from initial infection to the death of the patient is about two to ten years, while the duration of the more virulent Rhodesian form is only six to nine months. In the latter disease, there is less demarcation between the early and advanced stages of the infection, and the somnolence and other signs of nervous damage are rarely seen (Apted, 1970).

1.2.3 African Animal Trypanosomiases

Animal trypanosomes, particularly in cattle, are a major restraint on meat production in Africa. Four million square miles of

the African continent is infested with the tsetse fly (Buxton, 1955) and productive cattle cannot be kept within that area owing to the threat of trypanosomiasis (Willet, 1970). Wilson et al (1963) has suggested that if the tsetse fly were eradicated, the total head of cattle would be doubled. This would not only improve the nutrition of the African people, but also encourage the spread of agriculture by providing draught animals for ploughing. However, the eradication of the tsetse fly would not necessarily eradicate the disease, because the major pathogenic species (*T. congolense; T. vivax*) are known to be also transmitted by mechanical inoculation (Hoare, 1970).

In addition, Ormerod (1976; 1979) has argued that elimination of trypanosomiasis would be accompanied by overstocking of cattle, leading to ecological degradation (soil erosion) particularly in the Sahel region south of the Sahara; thus encouraging desertification. In contrast, Na'isa (1977) considers that as diseased animals are unproductive, trypanosomiasis encourages overstocking and, consequently, elimination of disease would solve this problem.

Cattle trypanosomiasis is primarily caused by *T. congolense*, *T. vivax* and *T. brucei*. Wild game serve as reservoirs for these trypanosome (Willet, 1970) and, as with *T.b. rhodesiense*, the trypanosomiases do not produce disease in their reservoir hosts (Ashcroft et al., 1959). *T. congolense* and *T. vivax* are the most important cause of nagana. The symptoms of the disease are mild fever, wasting and anaemia (Stephen, 1970). Death can occur within three weeks of infection with a virulent strain, although less virulent strains can cause chronic disease from which the animal spontaneously recovers

Cattle tolerate infection with *T. brucei* and recover spontaneously, although the infection is fatal to horses, with death usually occurring several months after the initial infection.

T. evansi is the causative agent of surra, a disease of camels and horses which is prevalent throughout Southern Asia, South America and Africa north of the Sudan zone (Hoare, 1970). This trypanosome is closely related to *T. brucei*, from which it is thought to have evolved (Hoare, 1970), but has lost the ability to be transmitted by the tsetse fly. The disease is transmitted by mechanical inoculation by biting tabanid flies (Hoare, 1970).

T. vivax and *T. congolense* can also be transmitted by mechanical inoculation, thus enabling them to exist outside the range of the tsetse fly. *T. vivax* has been found in Mauritius (Hoare 1970), the Caribbean (Hoare 1970), and in South America (Shaw and Lainson, 1972).

1.2.4 Life cycle of the salvarian trypanosomes (see Figure 1.1)

Trypanosomes (trypomastigote form) are ingested by the tsetse fly with its blood meal when it feeds on infected animals. The parasites undergo a series of changes within the fly during the next 17-45 days (Hoare, 1970). The parasite rapidly multiples in the mid-gut of the fly, and gradually migrates towards the tsetse fly mouth parts. Eventually the parasites reach the salivary glands, where they become attached to the wall of the gland and multiply further as the epimastigote form. Twenty to thirty days after ingestion, metacyclic forms appear and these are infective to the mammalian host (Hoare, 1970). Metacyclic forms are injected into the bloodstream of an animal on which the fly is feeding along with salivary secretion. After the subcutaneous

deposition, the trypomastigotes multiply and invade blood, lymph and tissues. Subsequently, the infection spreads to all tissues although, initially, the parasites do not seem able to reach or maintain themselves in the cerebro-spinal fluid (Apted, 1970). Three forms of trypomastigote develop in the bloodstream of the infected animal; long slender forms, intermediate forms and short stumpy forms. The short, stumpy form is believed to be the form which infects the tsetse fly (Vickerman, 1965).

The life cycle of *T. congolense* is slightly different in that the epimastigote and infective trypomastigote forms develop in the proboscis of the fly rather than the salivary glands, and the bloodstream form of the trypanosome is not pleomorphic (Hoare, 1970).

Trypanosoma vivax follows a different life cycle, as all of the development changes occur in the proboscis of the fly (Hoare, 1970). This trypanosome is often transmitted mechanically by biting flies and the parasites probably do not undergo developmental changes in this case. The bloodstream forms of *T. vivax* are monomorphic although virulent strains tend to be shorter than less virulent strains (Fairbairn, 1953).

Different developmental stages in the life cycle of trypanosomes have markedly different morphology and biochemical activity. In *T. brucei* the long slender forms of the parasite which are found in the vertebrate host have a DNA containing kinetoplast continuous with a simple mitochondrion containing almost no cristae and energy production is by glycolysis. When the trypanosome changes into the short stumpy form, which is infective to the tsetse fly, the mitochondrion proliferates and develops cristae, which are thought to be

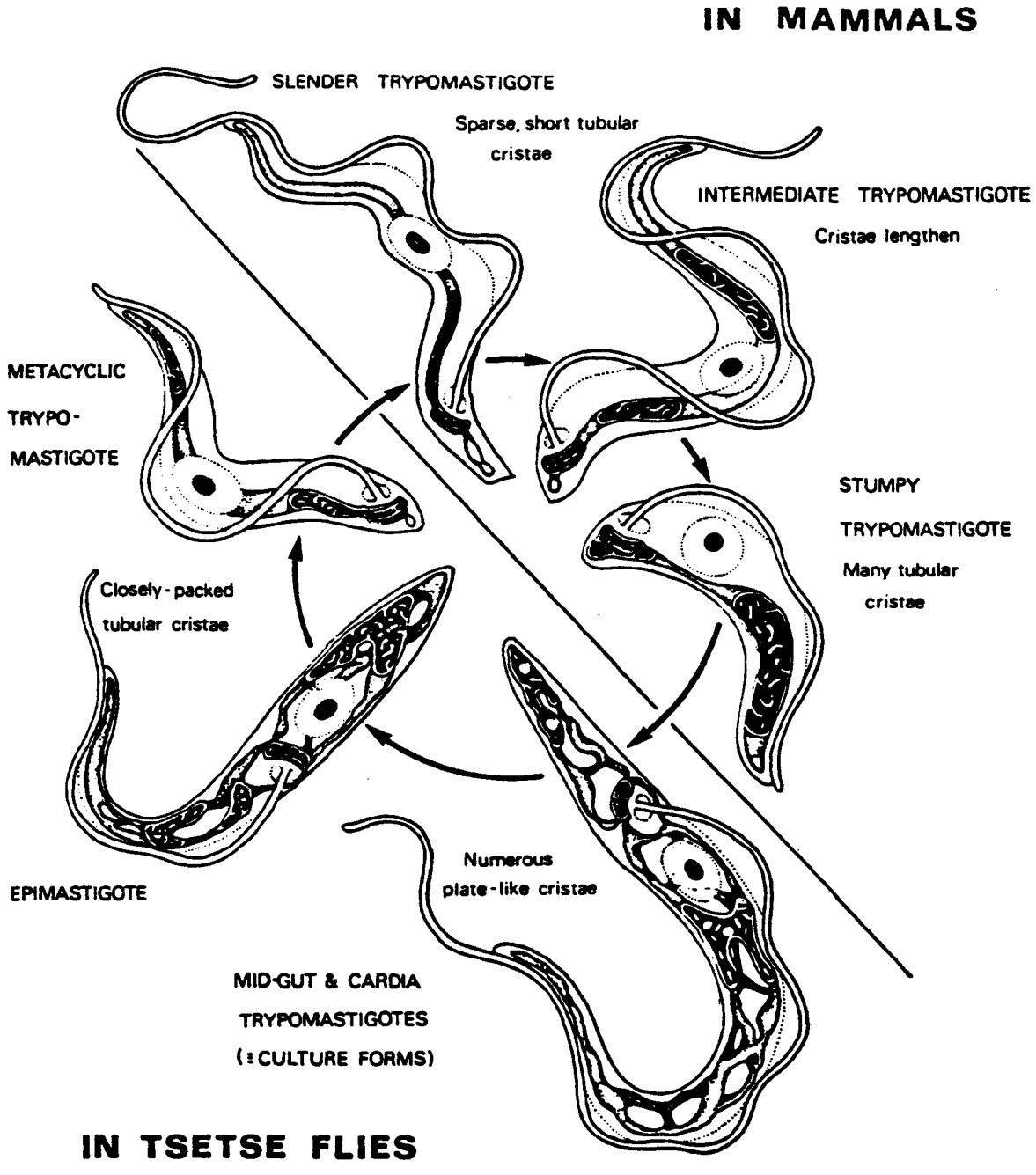


Figure 1.1: Life cycle of the *T. brucei* subgroup of trypanosomes (Vickerman, 1965).

a preadaption necessary for survival in the insect mid-gut. Once in the tsetse mid-gut energy production is by a citric acid cycle and a respiratory cytochrome chain (Vickerman, 1965).

An appreciation of the life cycle and biochemistry of the trypanosomes and the differences between host and parasite biochemistry is of fundamental importance in the rational design of trypanocidal drugs since these differences can be targets for chemotherapeutic attack.

1.2.5 American Human Trypanosomiasis

American human trypanosomiasis is caused by the stercorarian trypanosome *T. (schizotrypanum) cruzi*. The parasite was originally discovered by Carlos Chagas in Brazil (Lewinsohn 1979). The parastite has since been found in most countries of South America and as far north as Texas, USA.

It is difficult to assess the prevalence of the disease. The World Health Organisation (1967) estimate of seven million people infected is probably an underestimate, and the true figure is thought to be nearer 13-14 million (Zeledon and Rabinovich 1981).

The disease was originally a zoonosis and is prevalent in many wild and domesticated animals (Zeledon, 1974). Some of the insect vectors have become adapted to a domestic environment, and so the disease has spread to man (Zeledon and Rodriguez, 1981). The disease is transmitted by haematophagous triatomine bugs (Reduviidae family) which infest man's dwelling places. Transmission usually occurs through contamination of a wound or abrasion with excrement from an infected vector. Other modes of transmission have been discovered, such as congenital (Howard, 1962), through blood transfusion

or by contaminated food (Brener, 1971).

Many accidental infections of laboratory workers concerned with this parasite have also been recorded (Hawking, 1963).

1.2.6 Clinical features of Chagas disease

Chagas' disease is characterised by an acute and a chronic phase, although identification of patients with the acute phase is relatively rare even in endemic areas (Marsden, 1971). The acute phase is characterised by trypanosomes in the blood which are detectable by microscopic examination. Onset of symptoms begins about ten days after the initial infection and trypanosomes can be detected after about two to four weeks (Marsden, 1971). The acute phase lasts from six to ten weeks and is characterised by fever, oedaema, lymphadenopathy and cardiac irregularities (Brener, 1971). Ninety per cent of patients survive the acute phase, but it is doubtful whether their infection is ever eradicated (Marsden, 1971).

After one to two months, the parasitaemia subsides and the patient enters the chronic phase of the disease. During the chronic phase, the disease is asymptomatic in the majority of patients. Those patients who do manifest the disease overtly usually present one of two clinical symptoms which are chronic chagasic cardiomyopathy and the mega syndrome (Marsden, 1971). In chronic chagasic cardiomyopathy the heart is enlarged and cardiac contractions are weak. Intractable heart failure and sudden death associated with acute cardiac arrest, or ventricular fibrillation, are common (Marsden, 1971). The mega syndrome is caused by dilation of hollow muscular organs, such as the oesophagus and colon (Koberle, 1974). Although not commonly a cause of death, these deformations cause serious discomfort to the patient. The pathogenesis

of both forms could be related to the destruction of nerve cells in the gut and cardiac autonomic nervous system (Koberle, 1974), due to the formation of intracellular amastigote forms of the parasite.

Trypanosoma cruzi differs fundamentally from the African trypanosomes in many respects. These include mode of transmission, vectors, life cycle (especially intracellular invasion) and biochemistry. As this thesis is concerned with *T. rhodesiense*, a causative agent of sleeping sickness, discussion of *T. cruzi* will be included only where it is relevant.

1.3 Chemotherapy of Trypanosomiasis

Atoxyl (Figure 1.2) was the first successful trypanocidal drug to be introduced. It was found to be effective in early Gambian sleeping sickness, but although its use was sometimes followed by ocular atrophy, it did provide hope to sufferers from this previously incurable disease. The success of Atoxyl led its originator, Paul Ehrlich, to prepare a series of homologues leading to the discovery of salvarsan and ultimately tryparsamide (Figure 1.2) (Williamson, 1970). Tryparsamide was the first effective drug against the advanced stage of Gambian sleeping sickness and was the drug of choice for nearly 50 years after its introduction.

By 1950, arsenical resistant *T.b. gambiense* was widespread, and about 80% of late stage infections in the Congo were resistant to tryparsamide (Friedheim, 1949). Tryparsamide was superseded by the melaminyl series of arsenical drugs which were developed by Friedheim and based on the structure of the parent drug melarsen (Friedheim, 1941),

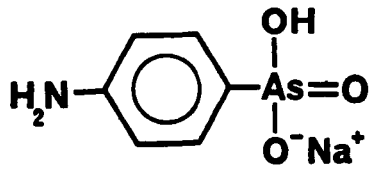
(Figure 1.2). Despite reports of toxicity, melarsen was soon followed by melarsoprol (Mel B), melarsonyl potassium (Mel W) and the antimony analogues MSb and MSb B (Williamson, 1970).

Of these drugs, melarsonyl has become firmly established in the treatment of Rhodesian sleeping sickness while melarsen and melarsonyl potassium are still used in parts of West Africa (Apted, 1970). Melarsoprol has proved effective against tryparsamide resistant Gambian and Rhodesian sleeping sickness at all stages of the disease (Van Hoof, 1947; Apted, 1953) and after its introduction in Tanzania, the death rate dropped from over one third to 10% of those who contracted sleeping sickness (Apted, 1957).

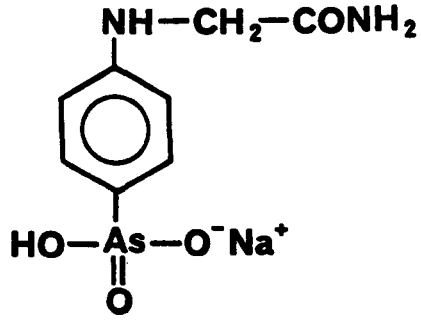
As well as development of the organic arsenical drugs, Paul Ehrlich was also interested in the use of organic dyes as potential trypanocides (Williamson, 1970). This line of enquiry stemmed from the observation that methylene blue, a cytochemical stain which was taken up avidly by the malaria parasite was also cytotoxic.

Following this discovery, Ehrlich tested many different dyes and eventually found one sulphonated azo dye: trypan red, which was both curative and prophylactic (Williamson 1970) in the treatment of trypanosomiasis. Modification of trypan red produced other compounds of greater activity, although none came into field use. Following from Ehrlich's work, chemists at Bayer in Germany prepared analogues closely related to Ehrlich's dyes, and their work culminated in the discovery of suramin (Bayer 205, Germanin) in 1916 (Williamson, 1970).

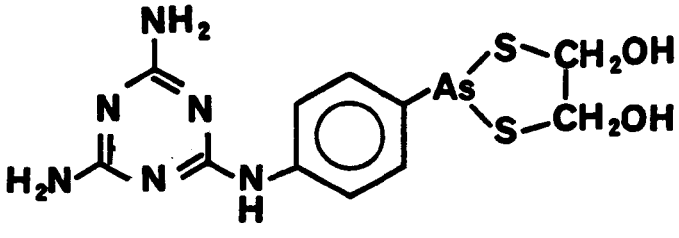
Suramin (Figure 1.3) is effective against the early forms of both Gambian and Rhodesian sleeping sickness with a cure rate of almost 100% (Apted, 1970). However, it will not pass from the blood into the



Atoxyl

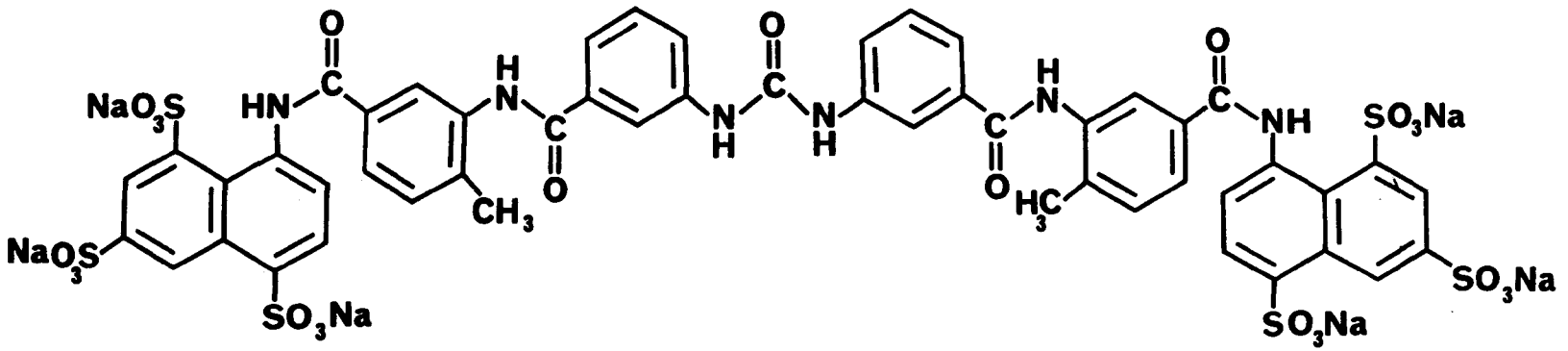


Tryparsamide

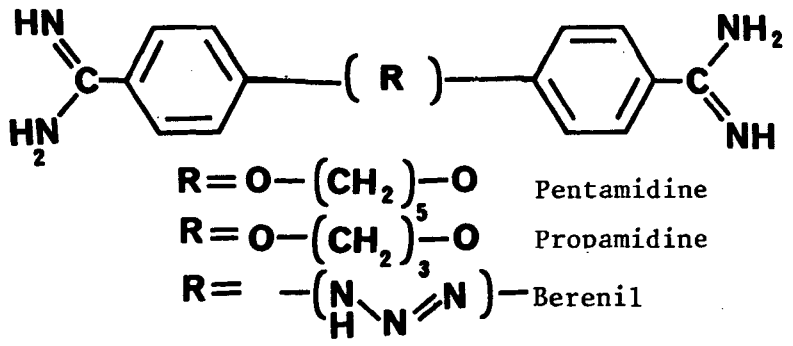


Melarsoprol (Mel B)

Figure 1.2: Structure of atoxyl, tryparsamide and melaroporol (Mel B)



Suramin



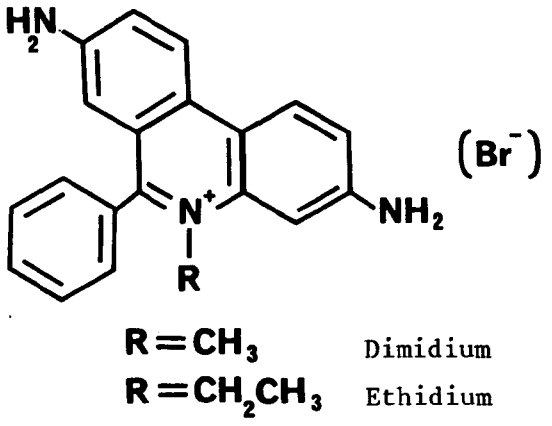


Figure 1.3: Structure of suramin, diamidine and phenanthridinium drugs

cerebro-spinal fluid and is of no use once the infection has reached the central nervous system, although it will clear trypanosomes from the blood and render the patient non-infective. Suramin combines with blood proteins and is excreted very slowly. A single injection of one gram will maintain a blood concentration sufficient to provide protection from infection for several months (Apted, 1970).

The trypanocidal activity of the diamidine series of drugs was discovered independently by Jancso and Jansco and Shern and Arlagaveytia-Allende in 1935 who observed that the aliphatic diguanidine synthalin, an insulin substitute, was trypanocidal *in vivo*. Further work by King et al. (1937), and May and Baker Ltd., produced several active aromatic diamidine derivatives, of which only pentamidine and berenil (Figure 1.3) have come into field use for trypanosomiasis. Pentamidine is a valuable drug for the treatment of early sleeping sickness and particularly as a prophylactic. A single dose of 300 mg will give complete protection for six months (Waddy, 1970). Berenil (diaminazene aceturate) is active against sleeping sickness, but is licensed only for veterinary use and is important in the treatment of nagana. Unlike pentamidine, berenil is rapidly excreted, usually within twenty-four hours of treatment. Although there is evidence that some of the drug is retained in the tissues (Williamson 1970) the rapid excretion of berenil is thought to lessen the risk of resistance developing through exposure to a waning concentration of drug; it also reduces the likelihood of chronic toxicity (Leach and Roberts, 1981).

The phenanthridinium drugs (Figure 1.3) were introduced by Morgan and Walls in 1931). Dimidium showed high activity against infections in cattle due to *T. vivax* and *T. congolense* (Williamson, 1962), but

treatment was often followed one to two months later, by severe toxic photosensitization and liver lesions. Ethidium, which differs from dimidium by having an ethyl group in place of a methyl group on the quarternary nitrogen atom, was as effective, and much less toxic (Leach and Roberts, 1981). Following the success of ethidium, other phenanthidinium drugs, most notably prothidium (Watkins and Woolf, 1952), metamidium and isometamidium (Wragg et al., 1958), have been introduced. No new trypanocide for human or veterinary use has been introduced since Mel B and metamidium, although research has continued. Williamson (1976) attributes this situation to the high cost involved in developing and marketing new drugs for countries which have low drug budgets, and are of uncertain economic stability. The World Health Organisation is attempting to overcome this problem by providing support for research through the Special Programmes for Research and Training in Tropical Diseases.

The need for new drugs for the treatment of trypanosomiasis is becoming more urgent as reports of drug resistance and problems of drug toxicity become more widespread. Trypanosome strains resistant to each of the main classes of drug and in some cases cross-resistant to several drug classes, have been reported (Williamson, 1976). De Raadt (1975) reported the appearance of a strain of *T.b. rhodesiense* which is resistant to melarsoprol. Many of the drugs currently in use, like melarsoprol, have a low therapeutic index, and the use of higher doses to combat resistance may be attended by toxic reactions. For example, melarsoprol, which is vital for the treatment of advanced Rhodesian sleeping sickness, causes fatal haemorrhagic encephalopathy in about 1% of patients when a standard dose is given (Apted, 1970).

It is thus too toxic to treat resistant strains, and alternative treatment, such as nitrofurazone, is also a toxic hazard.

1.3.1 Development of new trypanocidal drugs

Over the previous twenty years, three methods of research into new trypanocidal drugs have emerged. These are by (i) modification of existing drugs; (ii) empirical screening, and by (iii) rational consideration of the biochemical differences between parasite and host.

1.3.1(a) Modification of existing drugs

The organic arsenicals and the diamidines have provided many compounds of high intrinsic activity. Recently, Dann and co-workers have synthesized a series of diamidines (Dann et al., 1970; 1971; 1972; of which one, 6-amidino-2(4-amidinophenyl)-indole (DAPI) (Figure 1.4) has activity equivalent to berenil in experimental infections. However, resistance to one diamidine confers resistance to other drugs in the series and berenil resistant trypanosomes are also resistant to DAPI (Williamson, 1976). The possibility of cross-resistance to different drugs of the same type is a major weakness in this method of seeking new drugs. Many of the drugs now in use, such as metamidium, have been developed by combining portions of known trypanocides. This process of 'hybrid synthesis' (Williamson, 1962) is thought to favour the development of cross-resistance between drugs of different classes (Leach and Roberts, 1981).

1.3.1(b) Empirical Screening

This approach has revealed many new trypanocidal compounds particularly among antibiotics and drugs developed as antitumour agents,

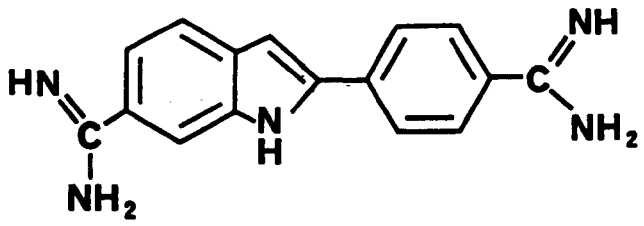
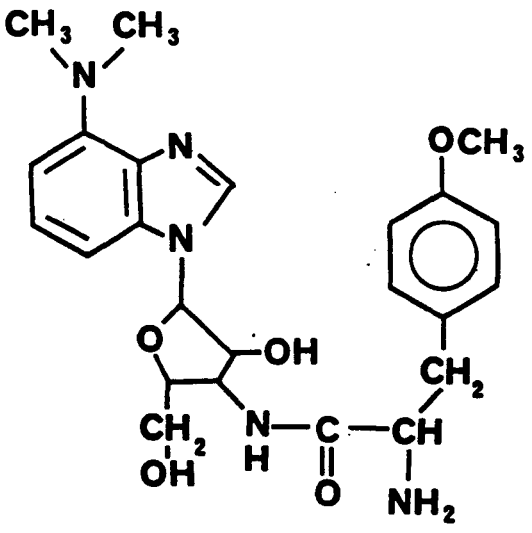


Figure 1.4: D.A.P.I. (6-amidino-2(4-amidinophenyl)-Indole

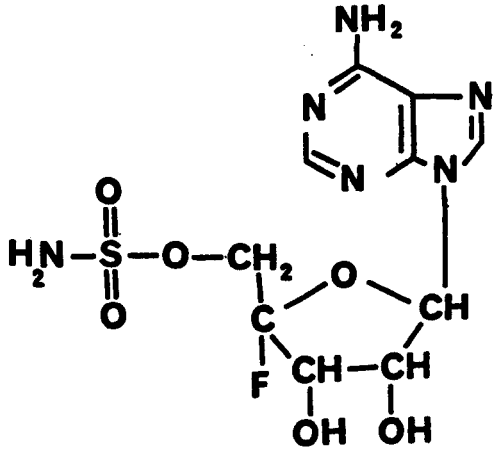
and antibiotics from *Streptomyces* species (Kinnamon et al., 1979; Williamson and Scott-Finnigan, 1975). A number of purine nucleoside analogues of adenosine, including puromycin and cordycepin (Figure 1.4) possess both antitumour and trypanocidal activity. This trypanocidal activity is significant as pathogenic African trypanosomes are unable to synthesise the purine ring and are dependent on their host for a source of preformed purine (Gutteridge and Gaborak 1979) One such analogue, puromycin (Figure 1.5) has been tested clinically and found to be effective in the early stage of sleeping sickness, but not when the central nervous system is involved (Williamson 1962) Metabolic analogies between pathogenic trypanosomes and rapidly dividing tumour cells are reflected in their similar sensitivity to drugs. Kinnamon et al. (1979) tested a range of 49 antitumour drugs against *T.b. rhodesiense in vitro* and found that six structurally unrelated drugs were trypanocidal; a higher proportion than would be expected by chance.

1.3.1(c) Rational approach

The lack of discovery of new drugs during the past twenty-five years has encouraged an investigation of the biochemistry of trypanosomes in order to identify and exploit the biochemical differences between parasite and host. Trypanosomes of the *T. brucei* subgroup, for example, show marked biochemical differences from the mammalian host. The bloodstream forms of *T. brucei* lack catalase (Fulton and Spooner, 1956), a tricarboxylic acid cycle, and enzymes involved in the biosynthesis of fatty acids (Bowman and Flynn, 1976). They also contain enzymes of unusual properties, such as a glycerol phosphate oxidase (G.P.O.) which, unlike G.P.O. in most eukaryotes, is



Puromycin



Nucleocidin

Figure 1.5: Trypanocidal nucleoside antibiotics

insensitive to cyanide, but is inhibited by salicyl hydroxamic acid (Fairlamb and Bowman, 1977). Following a study of the metabolism of the bloodstream form of *T. brucei*, Clarkson and Brohn (1977) discovered that S.H.A.M. in combination with glycerol totally inhibited trypanosome glycolysis and was trypanocidal *in vivo*. The development of this trypanocidal drug combination would not have been detected in a conventional screen for trypanocidal drugs, as neither compound is trypanocidal on its own.

Trypanosomes are more susceptible to damage by reactive oxygen species than mammalian cells, as they do not possess catalase, which destroys hydrogen peroxide. To exploit this lesion, Meshnick et al. (1978) have demonstrated that haematoporphyrin, a compound which catalyses the homolytic cleavage of hydrogen peroxide via a Fenton type reaction to form the highly reactive hydroxyl radical, is trypanocidal *in vivo*. Another vulnerable target for chemotherapy is polyamine metabolism. Biosynthesis of polyamines in trypanosomes is thought to occur as with other cells by conversion of ornithine into putrescine by a decarboxylation reaction, then to spermidine and spermine by sequential addition of an aminopropyl group (Bacchi, 1981). The synthesis of polyamines in *T. brucei* can be inhibited by a difluoromethyl ornithine (D.F.M.O.). Administration of a 2% solution of D.M.F.O. in drinking water cured mice infected with *T. brucei* (Bacchi, 1981).

Although none of the trypanocides discussed above has so far come into field use because of impracticality or toxicity, these examples illustrate that the rational approach to the design of drugs has considerable potential for discovering new trypanocides.

1.4 The anthracycline antibiotics

The anthracyclines are a group of antibiotics produced by various *Streptomyces spp.* Their name is based upon their structural features they contain an anthraquinone chromophore within a hydrocarbon skeleton related to that of the tetracyclines. Anthracyclines occur naturally as glycosides or as aglycones known as anthracyclinones.

The first clinically effective anthracycline - daunorubicin (Figure 1.6)was isolated independently in 1963 from *Streptomyces peucetius* by Casinelli and Orrezi and from *Streptomyces coeruleorubidus* by Du Bost et al. Daunorubicin has few clinical indications, but has achieved a significant role in the treatment of acute lymphocytic leukaemia and acute non-lymphocytic leukaemia (Bernard et al., 1969). Doxorubicin (Figure 1.6), a closely related antibiotic, was isolated from a mutant strain of *Streptomyces peucetius* (Arcamone, 1969); it is active against a wide spectrum of solid tumours, some of which were previously insensitive to chemotherapy (Carter, 1975). Doxorubicin has become established as one of the most clinically important anti-tumour drugs.

The anthracyclines are highly toxic, particularly to rapidly dividing tissues, and cause alopecia, stomatitis, myelosuppression and leukopaenia. The usefulness of daunorubicin and doxorubicin is limited by an irreversible and often fatal dose-related cardiomyopathy (Minow et al., 1975).

Although daunorubicin and doxorubicin differ only by the presence of a hydroxyl group at the C-14 position, they are clearly distinguishable at the cellular and clinical levels (Di Marco et al., 1974). The observation that a small modification in the chemical structure may produce substantial changes in the pharmacological

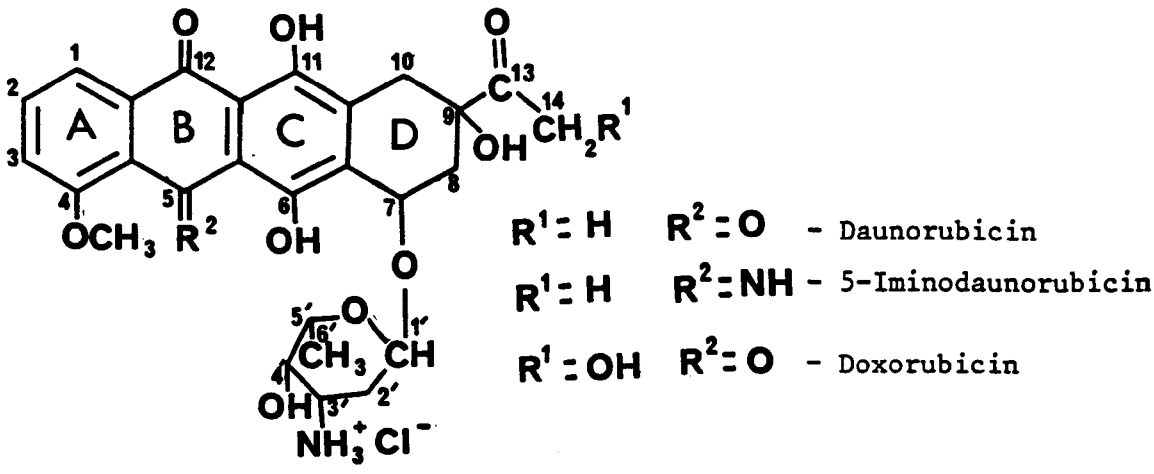


Figure 1.6: Structure of Daunorubicin, 5-Iminodaunorubicin and Doxorubicin.

properties of the drug has prompted the search for new anthracycline analogues with improved antitumour action and reduced cardiotoxicity. The main approach to the development of the anthracycline antitumour agents has been by chemical modification of the active anthracyclines (Arcamone, 1977; Brown, 1978). This approach has produced drugs with similar potency, but reduced cardiotoxic properties compared with daunorubicin and doxorubicin. Two examples of this approach are 5-iminodaunorubicin and N,N-dibenzyldaunorubicin (Acton et al., 1981).

Other important areas of research have been the isolation of new anthracyclines from microorganisms (Nettleton et al., 1980), and the development of synthetic anthraquinone analogues modelled on daunorubicin (Brown, 1983).

1.4.1 Anthracyclines as trypanocidal agents

These developments in anthracycline chemistry may be of importance to the chemotherapy of trypanosomiasis because of the established parallel between antitumour and trypanocidal activity (Kinnamon et al., 1979). The first demonstration of anthracycline activity against trypanosomes was the isolation by Fleck et al. (1972) of the anthracycline trypanomycin, which possesses trypanocidal activity against *T.b. brucei* in mice. The *in vitro* trypanocidal activity of the anthracyclines was also demonstrated by Williamson and Scott-Finnigan using *T. congolense* (1975), *T.b. brucei* and *T.b. rhodesiense* (1978). All of the anthracyclines tested were active, although daunorubicin was unique in being ranked, with nucleocidin, as one of the most potent trypanocides known (Williamson and Scott-Finnigan, 1978). Unfortunately, these workers showed both doxorubicin and daunorubicin to be inactive *in vivo* (Williamson et al., 1981).

1.4.2 Mechanisms of anthracycline cytotoxicity

Daunorubicin and doxorubicin are known to have many different cellular effects, but the major cytotoxic effect is thought to be inhibition of nucleic acid synthesis by interaction with nuclear DNA. This view is supported by a large body of data; for instance, autoradiographical and fluorescence quenching experiments which have shown that daunorubicin is concentrated in the nucleus of normal (rat liver) and tumour cells in culture (Bernard et al., 1969).

Daunorubicin and doxorubicin bind to DNA by a process of intercalation whereby the flat anthraquinone moiety inserts between adjacent base pairs (Figure 1.7) so that the D ring of the daunorubicin chromophore protrudes into the major groove of the DNA double helix (Quigley et al., 1980).

Intercalation of the drug into DNA causes changes in the properties of both DNA and rug which may be used to study the interaction. Upon intercalation, the adjacent base pairs move apart to accommodate the drug molecule. The resulting local distortion is relieved by a local unwinding of the double helix and a slight lengthening of the DNA molecule (Freifelder, 1971).

The anthracyclines, in common with other intercalating agents, such as lucanthone and ethidium, produce double and single stranded DNA breaks (Kohn and Zwelling, 1981). These strand breaks do not appear to be significant for cytotoxicity as Zwelling et al. (1982) found no positive relationship between cytotoxicity and production of strand breaks for the anthracyclines doxorubicin and 5-iminodaunorubicin.

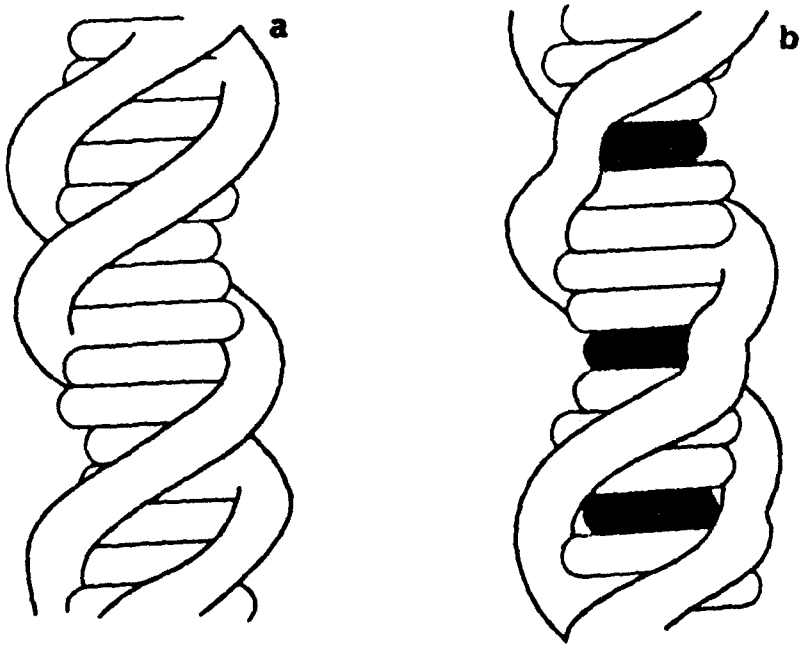
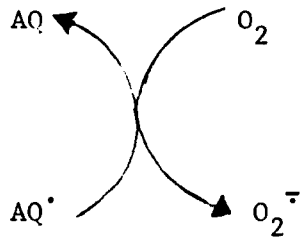


Figure 1.7: Intercalation of drug into the DNA double helix: (a) and (b) represent DNA before and after intercalation.

Daunorubicin and doxorubicin can also cause DNA strand scission through catalysing the formation of the superoxide anion. In the presence of NADPH and either microsomes or isolated nuclei, daunorubicin is reduced to the semiquinone anion (Bachur et al., 1978; 1982). The reduced anthraquinone moiety can then reduce molecular oxygen to the superoxide anion (Goodman and Hochstein, 1977) according to the following redox couple:



Detection of the semiquinone anion in culture cells treated with daunorubicin indicates that a similar process may occur *in vivo* Sato et al., 1977).

Hydrogen peroxide can be formed intracellularly by spontaneous dismutation of superoxide anion, or by the action of the enzyme superoxide dismutase (S.O.D.)

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow[\text{dismutase}]{\text{Superoxide}} H_2O_2 + O_2$$

Production of superoxide and hydrogen peroxide can lead, by a chelated iron catalysed reaction, to the generation of the highly reactive hydroxyl radical (McCord and Day, 1978)

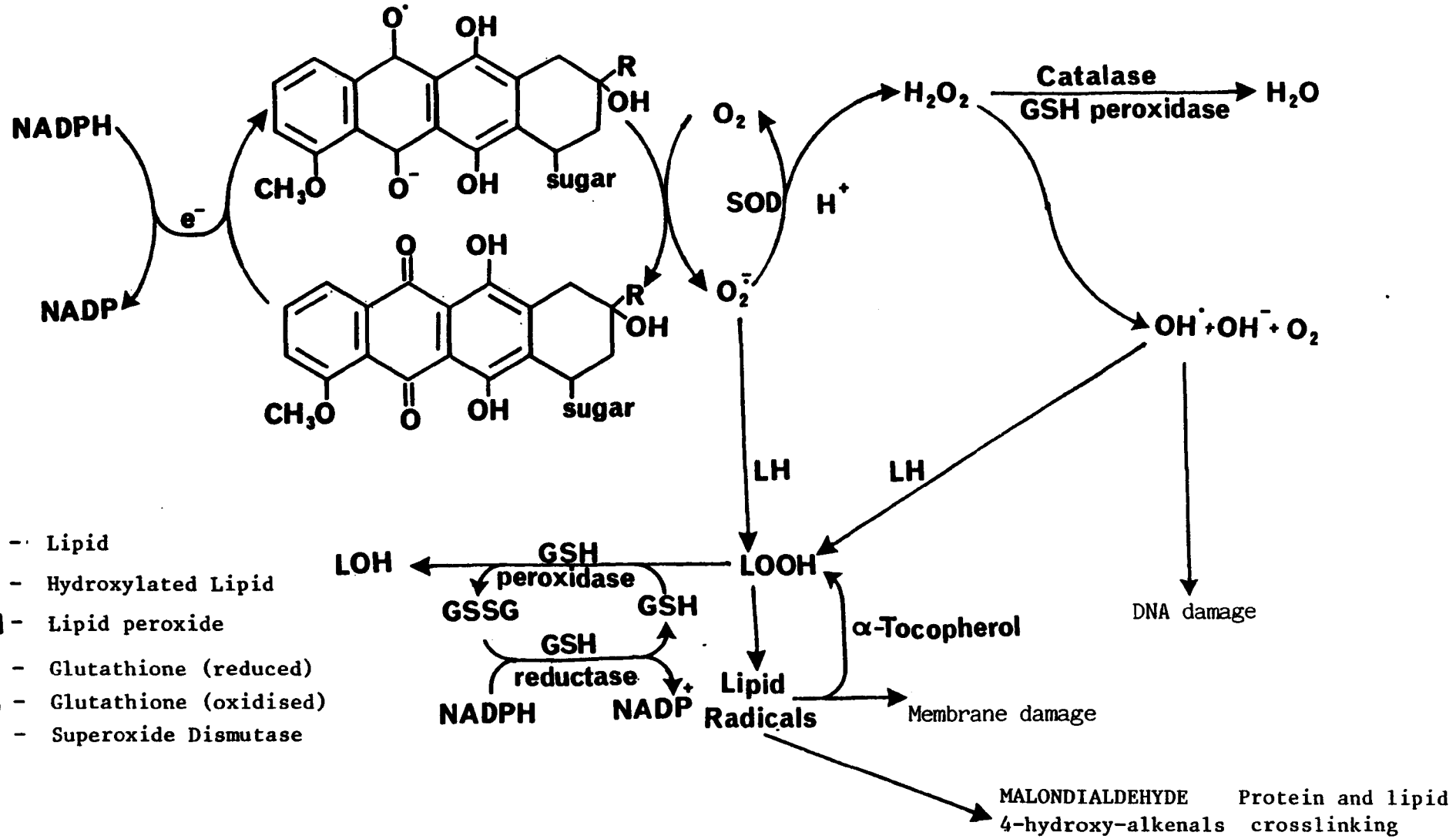
$$O_2^{\cdot-} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{iron}} OH^{\cdot} + OH^- + O_2$$

The hydroxyl radical is known to cause DNA strand scission (Lesko et al., 1980). However, since intercalating agents which do not catalyze the formation of reactive oxygen species also cause DNA strand breaks, other mechanisms of strand breakage may be

important (Zwelling et al., 1982). The hydroxyl radical can also be formed by a reaction between hydrogen peroxide and the doxorubicin semiquinone anion (Winterborne, 1981), thus obviating the requirement for molecular oxygen. This mechanism of hydroxyl radical formation may be important in trypanosomes, since they are known to possess high intracellular concentrations of hydrogen peroxide (Meshnick et al 1977)

Bachur et al. (1982) has postulated that the semiquinone radical is formed in the nucleus and DNA damage occurs on intercalation. The observation that chemically reduced daunorubicin binds DNA supports this mechanism (Sinha and Chignell, 1979). However, DNA cleavage by chemically reduced daunorubicin is partially inhibited by superoxide dismutase and catalase (Lown et al., 1977), indicating a role for the superoxide anion, hydrogen peroxide and/or hydroxyl radical.

In addition to DNA damage, the free radical species produced by anthracycline redox cycling may attack other intracellular targets. Administration of daunorubicin or doxorubicin is accompanied by a dramatic increase in microsomal lipid peroxidation (Goodman and Hochstein, 1977; Mimnaugh et al., 1981a). Lipid peroxidation is the free radical mediated degradation of unsaturated lipids which results in the formation of a variety of lipid peroxidation products, including alkanes and various aldehydes, including the 4-hydroxy-alkenals and malondialdehyde (Esterbauer, 1982), (see Figure 1.8). The potential cytotoxic effects of lipid peroxidation include loss of integrity of lipid membranes, disintegration of the endoplasmic reticulum and mitochondrial membranes (Hogberg et al., 1973; Pasquali-Ronchetti et al., 1980), impairment of membrane bound



Key

LH	-	Lipid
LOH	-	Hydroxylated Lipid
LOOH	-	Lipid peroxide
GSH	-	Glutathione (reduced)
GSSG	-	Glutathione (oxidised)
SOD	-	Superoxide Dismutase

Figure 1.8: Schematic representation of anthracycline induced lipid peroxidation

enzymes (Mimnaugh et al., 1981b), and damage to nucleic acid (Summerfield and Tappel, 1981). 4-Hydroxyalkenals produced during lipid peroxidation are known to react readily with proteins containing thiol and amino groups (Esterbauer 1982), and this might contribute towards the cytotoxicity of the anthracyclines. Malondialdehyde in particular is a known genotoxic agent produced during lipid peroxidation, and as a product of free radical attack on DNA (Gutteridge, 1979), can also cause DNA damage (Bird et al., 1982).

Lipid peroxidation has been implicated as a cause of the myocardial toxicity associated with the clinical use of daunorubicin and doxorubicin. Studies with animal models have shown that administration of doxorubicin is associated with perturbations of the intracellular antioxidant control systems, including decreases in glutathione (Olsen et al., 1977), glutathione peroxidase and selenium levels (Revis and Marasic, 1978). Conversely, endogenous administration of radical quenching agents, such as ubiquinone (Choe et al., 1979), α-tocopherol (Myers et al., 1977) and superoxide dismutase with catalase (McGuinness et al., 1979) protect against this myocardial toxicity. Cardiac tissue is believed to be particularly at risk from lipid peroxidation as it is deficient in two important enzymic defences relative to other organs. Superoxide dismutase activity in rat heart is about 20% of the activity of rat liver (Van Hien et al., 1975) and rat heart catalase activity is 2% of rat liver catalse activity (Thayer, 1977).

Reactive oxygen species may play an important role in the trypanocidal activity of the anthracyclines, as they lack important enzymie defences against active oxygen species *Trypanosoma cruzi* and *Trypanosoma b. brucei* both lack catalase (Boveris and Stoppani,

1977 ; Fulton and Spooner, 1956), and glutathione peroxidase (Boveris et al., 1980; Meshnick et al., 1978), although *T. cruzi* does contain superoxide dismutase (Boveris and Stoppani, 1977) and an ascorbate linked peroxidase with low activity (Docampo et al., 1976). However, intracellular concentrations of hydrogen peroxide are much higher in trypanosomes than in mammalian cells. Meshnick et al. (1977) have reported an intracellular concentration of 70 mM in *T.b. brucei* which is approximately thirty times that found in rat liver. This apparent metabolic weakness of trypanosomes has been exploited by Meshnick et al. (1977), who showed that haematoporphyrin, a compound which catalyses the homolytic cleavage of hydrogen peroxide to the highly active hydroxyl radical, was curative for *T.b. brucei* infections in mice.

The trypanocidal activity of β-lapachone and nifurtimox, two drugs which are active against *T. cruzi* was thought to be due to drug induced production of superoxide anions, and hence hydrogen peroxide (Docamps et al. (1978, 1981) which might accumulate to cytotoxic levels since *T. cruzi* does not contain catalase. However, the sensitivity of the catalase containing trypanosomatid *Crithidia fasciculata* to nifurtimox is incompatible with this mechanism of action (Gutteridge et al., 1982). However, *Crithidia fasciculata* is insensitive to β-lapachone (Lopes et al., 1978).

The anthracyclines have other mechanisms of cytotoxicity in addition to those discussed. Recently, Tritton and Yee (1982) and Tokes et al. (1982) have shown that doxorubicin does not need to enter the cell in order to exert a cytotoxic effect. The mechanism by which this cytotoxicity occurs is unknown at present, although

doxorubicin has previously been shown to exert various effects on the cell membrane. These have included effects on phospholipid structure, membrane fluidity, transport of small molecules and expression of hormone and lectin receptors (Tritton and Yee, 1982).

1.5 The targeting of chemotherapeutic agents

The chemotherapy of both cancer and parasitic diseases has been compromised by the toxicity of the available drugs. A major problem is the lack of specificity of the drug for the target cell. This, as well as the lack of progress in developing new antiparasitic drugs, has stimulated research into ways of improving the therapeutic index of existing drugs. Considering tumour cells, metabolic differences between these and normal cells are frequently quantitative, rather than qualitative: the action which produces a desired effect in the tumour, will also produce severe toxicity to normal cells. Hence, the optimum dose of an antitumour agent is often a compromise between sub-optimal therapeutic effects and tolerable toxic effects (Poznansky and Cleland, 1979). Similarly, many of the antiparasitic drugs, such as the arsenicals and antimonials, also produce severe toxic effects which limit their clinical usefulness (Apted, 1970).

Improvement of the therapeutic index of existing drugs can be approached in several ways: (i) preparation of analogues by formation of a pro-drug with enhanced uptake properties for the target cell and/or greater metabolism of the pro-drug in the target cell compared with normal cells; (ii) binding of drug to macromolecular carrier; (iii) entrapment of free drug in an encapsulation carrier.

1.5.1 Pro-drugs

In order to rationally design a pro-drug, the biochemical and morphological characteristics of the target cell must be known so as to exploit differences between these cells and normal cells, and the choice of pro-drug derivative is also limited by the functional groups present in the parent drug which are suitable for derivatization. As regeneration of the parent drug is required, the choice of derivative is usually limited to groups possessing enzymic, or chemical lability, such as peptides and esters.

An example of the rational design of a pro-drug is a peptide derivative of phenylene-diamine mustard which Carl et al. (1980) have shown to be selectively hydrolysed by tumour cells to the parent drug.

An example of pro-drug design applied to the anthracycline series is rubidazone, a benzoyl hydrazone derivative of daunorubicin *in vivo* (Baurain et al., 1979).

Another example of pro-drug design applied to the anthracyclines is a series of peptide derivatives of daunorubicin developed by Masquelier et al. (1980). These pro-drugs were metabolised to daunorubicin by lysosomal peptidases, but were more active than daunorubicin against L1210 leukaemia *in vivo* (Baurain et al., 1980).

1.5.2 Macromolecular conjugates

The use of macromolecules to target drugs was first proposed by Paul Ehrlich in 1906 who predicted the existence of antibodies and foresaw their use as drug carriers. Macromolecules were first used as drug carriers by Mathe et al. (1953) who demonstrated the enhanced antitumour activity of methotrexate when covalently bound

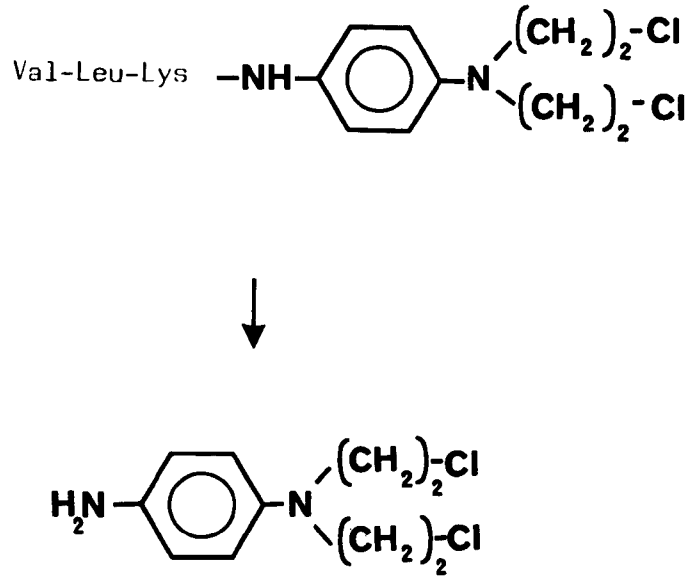


Figure 1.9: Pro-drug specifically activated by tumour associated enzymes (Carl et al., 1980)

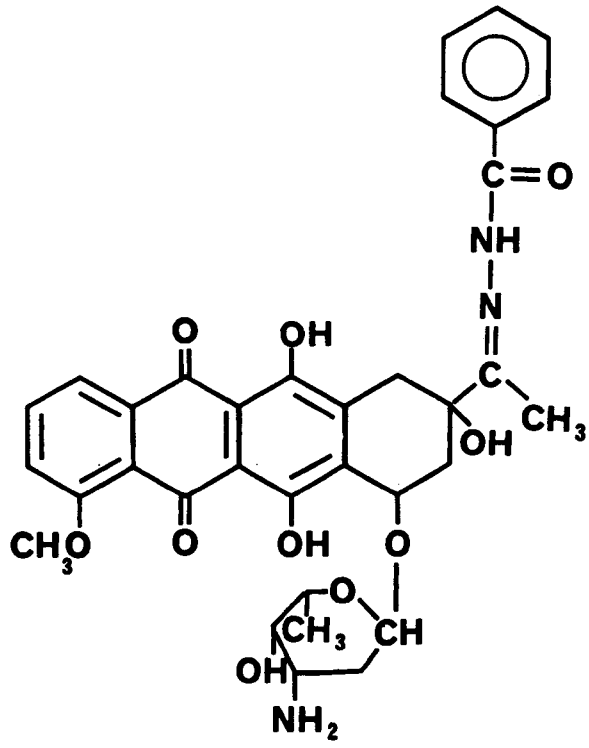


Figure 1.10: Rubidazone - a daunorubicin pro-drug

to tumour-specific antibodies. Subsequently, a variety of macromolecular carrier systems have been developed, both with and without specific affinity for the target cell. These have included macromolecules such as antibodies (Hurwitz et al., 1975), lectins (Kitao and Hattori, 1977; Lin et al., 1981) and hormones (Varga et al., 1977; Kaneko, 1981) which have specific affinity for the target tissues, albumin (Trouet et al., 1982) and dextran (Levi-Schaffer et al., 1982) which have no such affinity. As well as improving drug uptake into the target tissue, carriers have been used to enhance a variety of desirable characteristics of drugs and enzymes used in therapy. L-Asparaginase, an enzyme used in the therapy of acute lymphocytic leukaemia, is rapidly inactivated *in vivo* as well as producing a severe immunogenic side effect characterised by anaphylactic shock. Serum albumin has also been used as a drug carrier to reduce the rate of drug clearance from the body (Poznansky and Cleland, 1980). The formation of a non-covalent drug-albumin conjugate is an important factor in explaining the slow plasma clearance and the prophylactic activity of the trypanocidal drug suramin (Goldstein, 1949).

1.5.2(a) Mechanism of action of drug-macromolecule conjugate

The carrier requirements of different therapeutic agents will vary widely and, in practice, no carrier system will satisfy the requirements of a broad spectrum of drugs and enzymes. However, there are certain characteristics that must be considered in the rational design of an 'ideal' carrier. The primary requirement of

a carrier used for targeted antiparasitic chemotherapy is that it should reach the target tissue without breakdown, or inactivation, by plasma enzymes. Uptake by host tissues should be minimal and the carrier should not elicit an immune response.

Once transported to the vicinity of the target cell, the drug carrier conjugate may exert its effects in several ways. The first possibility is that the drug might be cleaved by degradative enzymes in the extracellular fluid, allowing free drug to permeate into the cell. This mechanism might well be important in cancer chemotherapy as lysosomal enzymes have been detected on the surface of tumour cells (Naoy et al., 1977) and in necrotic tumour areas (Strauli and Weiss, 1977). Another possibility is that the intact drug-carrier complex may enter the target cell by endocytosis, after which the endocytic vacuole fuses with a lysosome to form a secondary lysosome. The drug is released by lysosomal hydrolysis and may diffuse through the cytoplasm to its intracellular target. This mechanism has been studied by Deprez-De Campaneere et al. (1979) who demonstrated that the mechanism was valid for the release of drug from the doxorubicin-DNA complex.

Weisenhahn et al. (1981) showed inhibitors of endocytosis decreased the toxicity of daunorubicin-melanotropin conjugate for mouse melanoma cells, and also showed that the conjugate did not interact with DNA *in vitro*, which suggests that intracellular release of daunorubicin is necessary for activation of this conjugate.

The drug carrier complex may act intracellularly without preliminary drug release. At present there is little to support this hypothesis, but Hurwitz et al. (1975) have reported that daunorubicin linked to antibody can interact with DNA, and Lewis

et al. (1974) have shown that, following pinocytosis antibodies can be found associated with nucleus of human lymphocytes. The idea that anthracycline conjugates can be active in their own right has recently received support from the work of Tritton and Yee (1982) who showed that doxorubicin-linked macromolecules could exert cytotoxic effects without entering the cell.

1.5.2(b) The use of anthracycline-macromolecule conjugates

The use of anthracycline-macromolecule conjugates in the treatment of cancer was proposed by Trouet et al. (1972) who studied the use of daunorubicin-DNA and doxorubicin-DNA conjugates in the treatment of tumours possessing a high rate of endocytosis. Animal studies (Deprez-De Campaneere, 1979) have shown that these DNA conjugates are superior to the free drugs in the treatment of several different types of tumour.

A major obstacle to the widespread use of DNA conjugates is that they lack tissue specificity and are taken up by cells possessing a high rate of endocytosis. In order to overcome this problem, Hurwitz et al. (1975) have systematically studied covalently bound anthracycline-antibody conjugates. All of the conjugates tested possessed antitumour activity, but conjugates containing a high loading of drug had reduced antibody activity. The loss of activity which accompanies the formation of antibody conjugates has been a major problem in using antibodies as drug carriers. In an attempt to increase the amount of drug bound while retaining antibody activity, Bernstein et al. (1978) linked daunorubicin to dextran prior to linking the complex to antibody. This procedure allowed a five-fold increase in the amount of drug bound per conjugate molecule compared

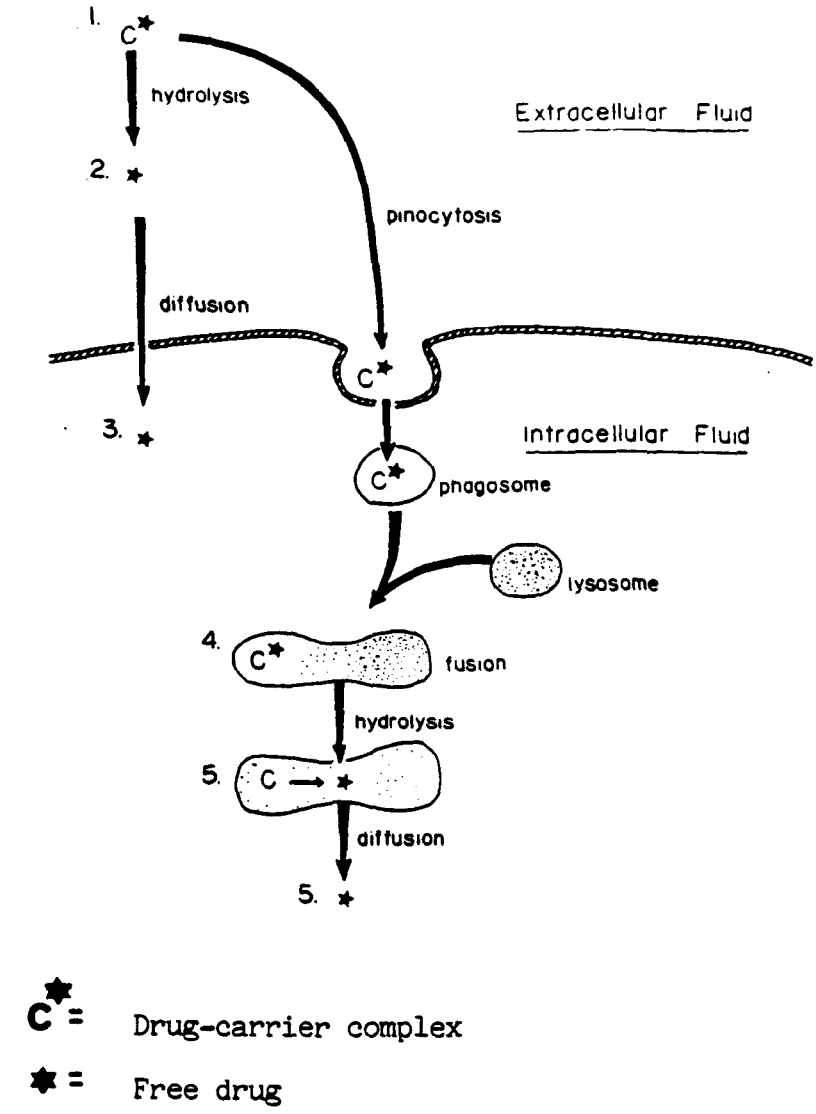


Figure 1.11: Modes of action of a drug-carrier complex.
(Poznansky and Cleland 1979).

The drug can enter the cell either by simple diffusion following extracellular release from the carrier or endocytosis of the drug-carrier complex. Once inside the cell, drug may be released from the drug-carrier complex by the action of lysosomal hydrolases.

to that linked to antibody alone. However, when tested *in vivo* the drug antibody conjugate had no therapeutic advantage over the equivalent dose of free drug, but was more active than drug bound to a non-specific immunoglobulin. The best results were obtained when drug and antibody were given simultaneously. This drug-antibody synergism has been noted with other drugs (Ghose and Nigara 1972)

Other site-specific carriers in addition to antibodies, have been used to target daunorubicin. Varga et al. (1977) have used melanotropin to target daunorubicin to mouse melanoma cells. They found that the conjugate was three times more toxic to melanoma cells compared to the free drug, although non-specific cells were unaffected. Kaneko (1980) has linked daunorubicin to the hormone thyrotropin and demonstrated that the conjugate possesses specific affinity to the thyroid cells. Concanavalin A, a lectin with specific affinity for tumour cells, has been evaluated as a site-specific carrier of daunorubicin. The conjugate cured mice bearing Ehrlich ascites tumour, but daunorubicin given alone had little effect (Kitao and Hattori, 1977).

1.5.2(c) Drug-macromolecule linkages

The link between carrier molecule and drug may be either covalent, or non-covalent, but most studies have been performed using a covalent type of conjugation. While the distribution of the drug is dictated by the characteristics of the carrier molecule, the type of linkage used has a fundamental effect on the physical and chemical properties of the conjugate and, hence, is of great importance in their design of drug carrier preparation.

In considering a suitable linking reaction, the drug and carrier functional group moieties available for reaction must be identified.

Linking agents which react with functionally important groups with respect to pharmacological activity on the drug or carrier molecule, may produce inactivation (Ghose and Blair, 1978). Drugs or carriers which lack suitable reactive groups may be modified to provide these groups. Steroids containing hydroxyl groups have been reacted with succinic anhydride to yield a hemisuccinate which is amenable to coupling by the carbodiimide method (Abraham and Grover, 1977). In the periodate oxidation method, vicinal-hydroxyl groups are oxidatively cleaved to yield aldehyde groups which can be used to form a Schiff's base linkage (Erlanger, 1973). The conditions under which the binding reaction occurs must be carefully considered, as the conditions required for some reactions may degrade the drug or carrier. Some reagents such as hexamethylene diisocyanate are insoluble in water, and require the use of non-aqueous solvent systems which would denature a protein carrier. Reagents such as cyanogen bromide and the alkyldiimido esters require strongly alkaline conditions (Poznansky and Cleland, 1980). The carbodimides and glutaraldehydes are the most extensively used cross-linking reagents and have been used to link a wide range of antitumour drugs to macromolecules (Hurwitz et al., 1975; Goodfriend et al., 1964) as well as linking haptens to proteins in order to elicit antibodies (Erlanger, 1973). These reagents have appreciable advantages over comparative cross-linking schemes. The reactions may be carried out in aqueous solution at neutral pH and at room temperature. They also utilise the abundant anions and carboxyl groups of protein. However, they may also cause protein cross-linking which gives denaturation and inactivation of the protein carrier (Erlanger, 1973).

Other cross-linking reagents have serious shortcomings which has precluded their widespread use. The cyanogen bromide, bisdiazobenzidine, and mixed anhydride methods (see Table 1.2) require the use of very toxic compounds, while other reagents, most notably diazo linkages and cyanogen bromide, react with groups that are of low abundance in protein carriers.

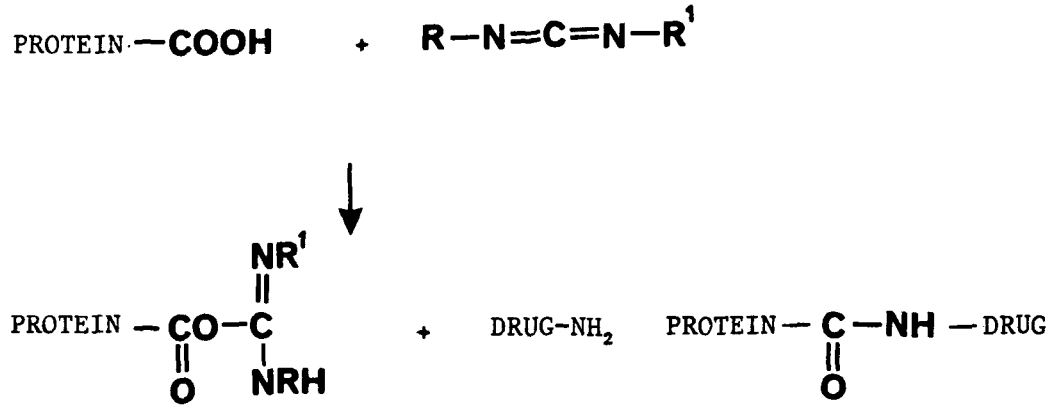
1.5.3 Encapsulation carriers

Encapsulation carriers are those in which the drug is physically separated from the surrounding environment. Carriers of this type, which have been utilised in chemotherapy, have included ghost erythrocytes (Lynch et al., 1980), polyalkylcyanoacrylate nanoparticles (Couvreur et al., 1982), albumin microspheres (Widder et al., 1981) and liposomes. Most research into the role of encapsulation carriers in chemotherapy has been using liposomes. This is because they are very versatile and their physical properties, such as drug loading, size, charge and composition, can easily be varied. Liposomal entrapment can change the pharmacokinetic properties and toxicity of the encapsulated drug. Juliano and Stamp (1978) have shown the liposomal encapsulation of daunorubicin increased the plasma half-life from five minutes to 150 minutes, and Rahman et al. (1982) demonstrated that liposomally encapsulated doxorubicin was only half as cardiotoxic as the free drug. Enhanced antitumour activity of liposomally entrapped doxorubicin in Ehrlich solid tumour-bearing mice has been demonstrated by Shinozawa et al. (1981) who showed that liposomal encapsulated doxorubicin inhibited tumour growth by 55% compared with free doxorubicin.

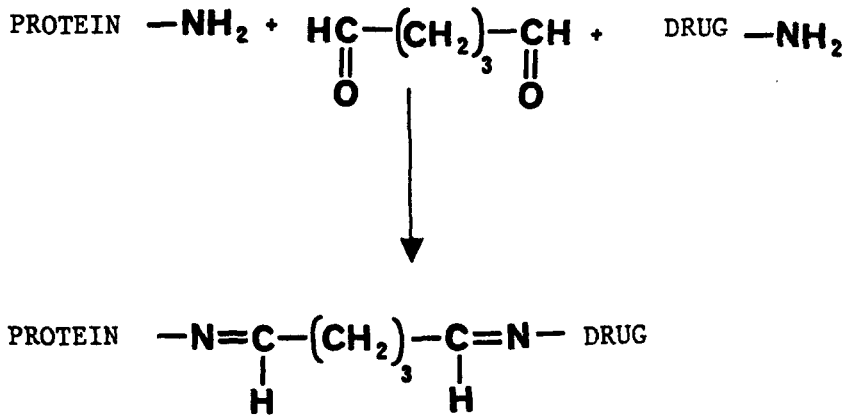
One obstacle to the widespread use of liposomally entrapped drugs is that they lack tissue specificity and are rapidly endocytosed by

Table 1.2: Methods of drug-macromolecule linkage

a) Carbodiimide



b) Glutaraldehyde



c) Diethylmalonimidate

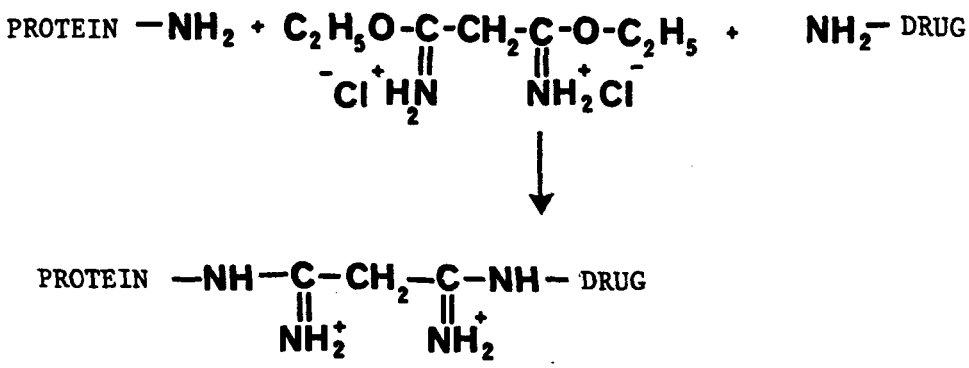
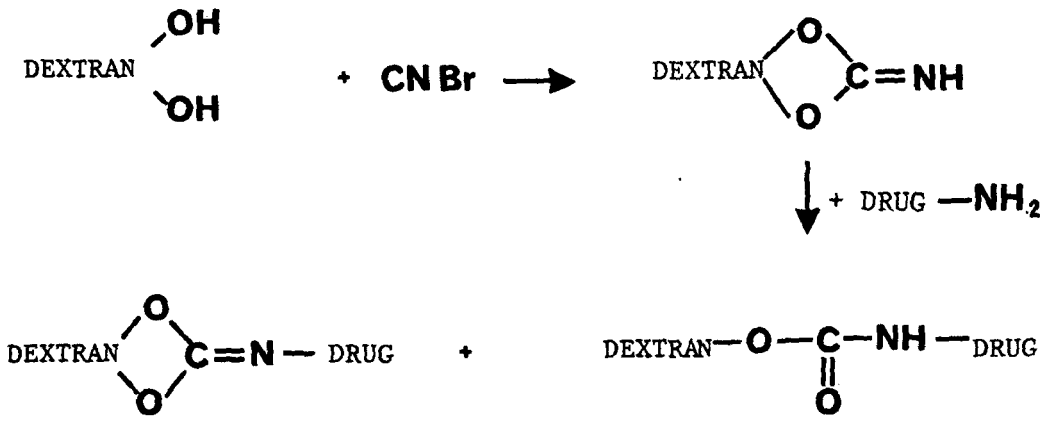
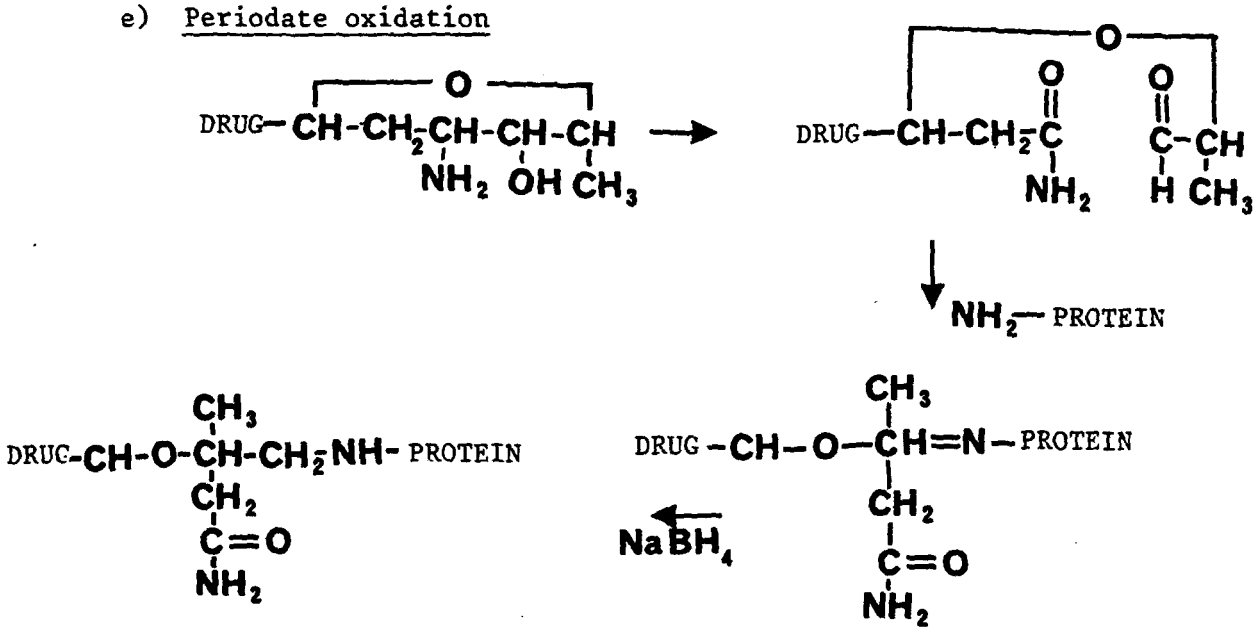


Table 1.2 (contd.)

d) Cyanogen bromide CNBr



e) Periodate oxidation



f) Diazo linkage

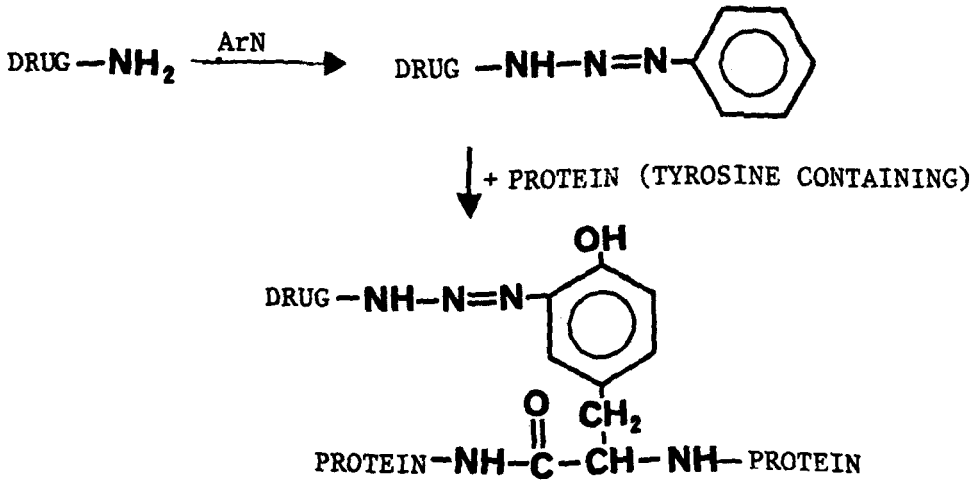
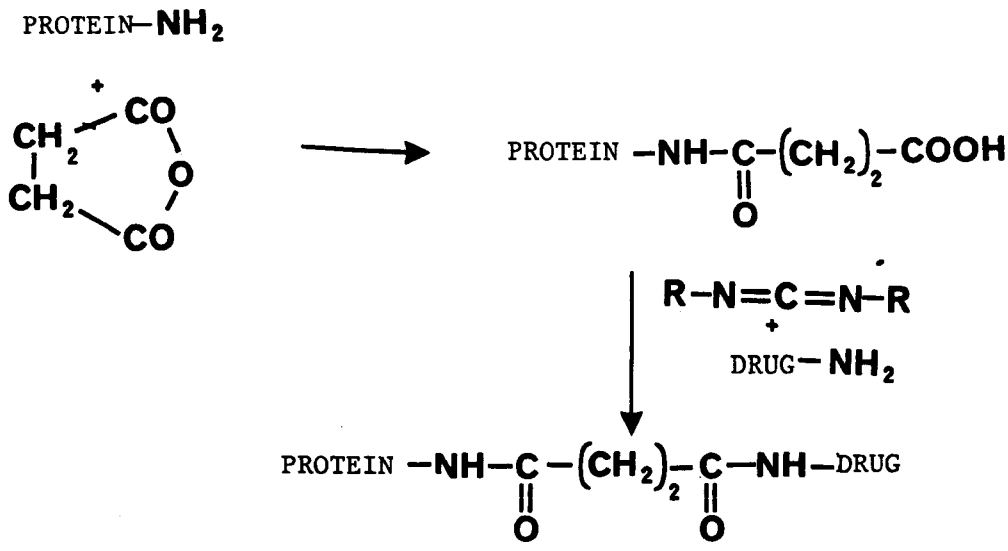
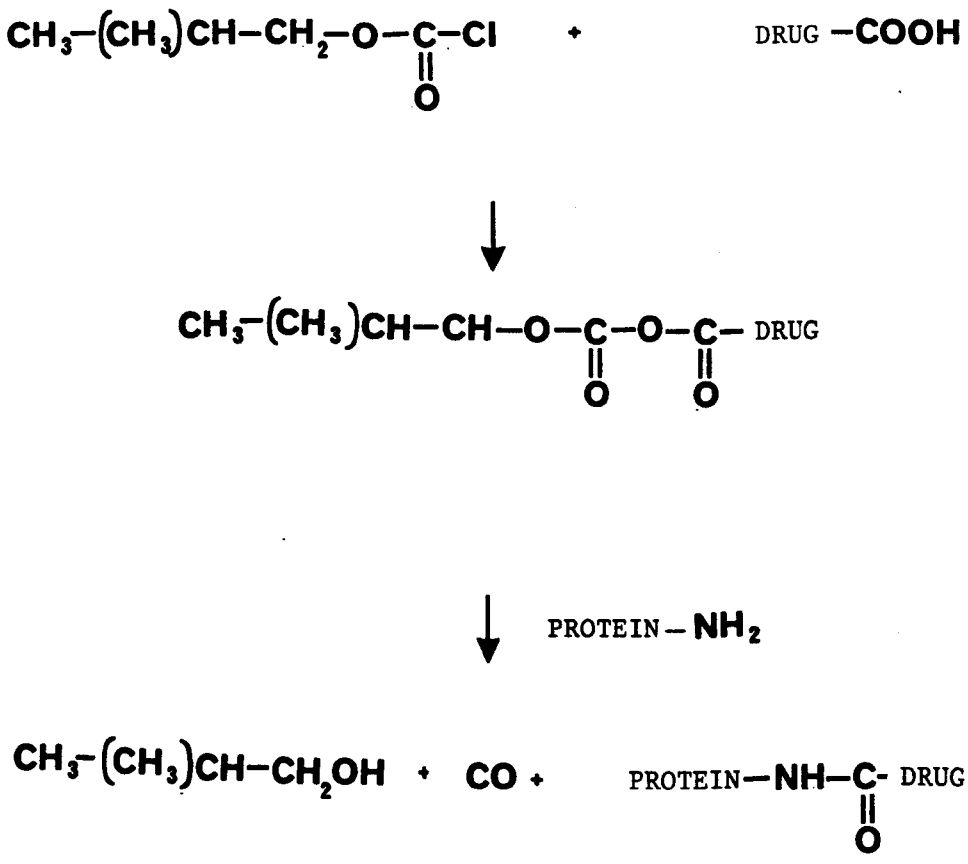


Table 1.2 (contd.)

g) Succinic anhydride



h) Mixed anhydride



by the reticuloendothelial cells of the liver and spleen. Research is in progress to covalently attach antibodies to liposomes (Gregoriadis et al., 1982) which would enable them to be targeted to specific tissues.

The enhanced uptake of liposomes by the reticuloendothelial system has been exploited in the treatment of the parasitic diseases leishmaniasis and malaria which reside in the liver and spleen (Alving, 1982). This use of liposomes is discussed in section 1.5.4.

1.5.4 Macromolecules as carriers of antiparasitic drugs

The important parasitic diseases leishmaniasis, malaria and chagas disease possess an intracellular stage in their lifecycle, which is difficult to treat by conventional drug therapy. This is thought to be due to the inability of drugs to reach the intracellular site of the parasite in cytotoxic concentration. Carriers have been successfully used in several cases to transport drug to the intracellular parasite. The intracellular (amastigote) form of *Trypanosoma cruzi* is refractory to drugs which kill the bloodstream (trypomastigote) form. Trouet et al. (1976) have demonstrated that administration of an ethidium bromide-DNA will cure mice infected with *T. cruzi*. Administration of free ethidium bromide increased the survival time of the mice tested, but did not affect any cures. However, Avila et al. (1979) confirmed the sensitivity of *T. cruzi* to ethidium bromide, but were not able to cure infected mice by administration of an ethidium bromide-DNA complex.

Liposomes have been used to target antimonial drugs to the intracellular parasite. *Leishmania donovani*, the causative agent of visceral leishmaniasis, resides preferentially in the reticulo-endothelial cells of the liver and spleen and is therefore vulnerable to liposome encapsulated drugs. Alving et al. (1978) have shown that the drug meglumine antimoniate is three hundred times more effective when incorporated into liposomes than when given alone.

Liposomes have also been used to target the antimalarial drug primaquine to the tissue schizonts of *Plasmodium berghei*, which invades hepatocytes. An increase in schizonticidal activity, and a decrease in toxicity were observed, although the effects were small. Liposomes have not been used at present to target drugs to trypanosomes of the *T. brucei* sub-groups, although Greenberg et al. (1979) has shown that positively charged fluid liposomes will fuse with the trypanosome cell membrane.

As the examples above have shown, carriers of various types possess considerable potential for improving the effectiveness of antiparasitic drugs.

1.6 Aims and objectives of the present investigation

New drugs are urgently required for the treatment of African trypanosomiasis. No new drugs have been brought into field use during the past twenty-five years; also, there are serious obstacles to the use of some drugs currently available, such as widespread resistance to the cattle trypanocides ethidium and antrycide, and the acute toxicity of some arsenical drugs such as melarsoprol (Williamson, 1976). The lack of new trypanocides has prompted the routine screening of known cytotoxic drugs for trypanocidal activity. Williamson and

Scott-Finnigan (1978) used a rapid *in vitro* assay to screen a series of antitumour agents for trypanocidal activity. Of the drugs screened, daunorubicin was found to possess uniquely high trypanocidal activity, permanently abolishing the infectivity of *T.b. brucei* at nanomolar concentrations and establishing itself as one of the most trypanocidal compounds known. However, daunorubicin is totally inactive against trypanosomes in infected mice (Williamson and Scott-Finnigan, 1978). This lack of *in vivo* activity may be considered to be due to either (a) daunorubicin not reaching the intracellular target in cytotoxic concentrations (pharmacokinetic factors), or (b) metabolic differences between the *in vivo* and *in vitro* forms of the parasite (metabolic factors).

In either case improved delivery of drug to the organism would perhaps circumvent the loss of activity *in vivo*. Linking of drug to a macromolecule would, in the case of daunorubicin, be expected to prolong the blood level of the drug since daunorubicin is rapidly cleared from the blood (Brown, 1983). Hence, increased exposure of the organism to the drug would be expected. Similarly, if the macromolecule could be endocytosed by the organism, then a greater uptake would be expected with a longer residence time in the organism. This coupling of daunorubicin to macromolecules was, indeed, successful in retaining activity *in vivo* as explained in the early sections of the research and discussion.

The characteristics of the daunorubicin-macromolecule conjugate which were required for activity were then systematically studied by (a) variation of the macromolecule, (b) variation of the linkage used and (c) measurement of drug release in biological fluids. These studies may provide a basis for rationally designed daunorubicin-macromolecule conjugates with improved properties.

The second phase of this study was to establish the mechanism by which daunorubicin exerts its trypanocidal effect. The cytotoxicity of daunorubicin is believed to be due to intercalation of drug into the DNA double helix (Henry, 1976), although other mechanisms such as toxicity mediated by reactive oxygen radicals may be important for trypanosomes in view of their lack of enzymic defence towards these species (Fairlamb, 1982). Measurement of the ability of a series of daunorubicin derivatives to intercalate into DNA, and to stimulate lipid peroxidation in biological material, may be compared with their trypanocidal activity, and thus may enable the trypanocidal properties of daunorubicin to be identified. This may be of use in developing drugs in which the trypanocidal properties of daunorubicin can be separated from other toxic properties of the anthracyclines.

The aims of the study were, therefore, (a) to modify daunorubicin so as to enhance uptake and overcome pharmacokinetic barriers to activity *in vivo*; (b) to study the metabolism of drug-macromolecule conjugate in biological fluids so as to establish the breakdown characteristics required for activity, and hence design a drug-macromolecule conjugate with optimum activity; (c) to investigate the trypanocidal mechanism of action of daunorubicin.

CHAPTER 2: EXPERIMENTAL METHODS

2.1 Synthesis of anthracycline conjugates

2.1.1 Materials

Daunorubicin hydrochloride ('Cerubidin' vials containing 20 mg drug plus 100 mg mannitol) was purchased from May and Baker (Dagenham, Essex, UK) and used without further purification. Pure daunorubicin hydrochloride was purchased from Sigma (Poole, Dorset, UK). 5-Imino-daunorubicin was a gift from Dr. E.M. Acton (Stamford Research Institute, California, USA). 4′-Demethoxydaunorubicin, 4-Deoxydaunorubicin, 3′,4′-diepidaunorubicin, doxorubicin and 4′-deoxydoxorubicin were gifts from Dr. F. Arcamone (Farmitalia, Italy).

Bovine serum albumin, histone F_1 horse spleen apoferritin and dextran were all purchased from Sigma (Poole, Dorset, UK) and horse spleen ferritin was purchased from Boehringer Mannheim (Lewes, Sussex, UK). Aqueous glutaraldehyde (25%) was purchased from British drug Houses (Poole, Dorset, UK), 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulphonate (C.M.D.T.) was purchased from Aldrich (Gillingham, Dorset, UK). All other materials were reagent grade or analytical grade where appropriate, and were used as purchased. Phosphate buffered saline (P.B.S.) was prepared as described in Appendix I. Glassware was silanised with dichlorodimethylsilane (Hopkins and Williams, London, UK), rinsed in methanol and distilled water, and dried before use. Infrared spectra were recorded on a Perkin Elmer model 198 spectrometer. Mass spectra were recorded on a V.G. Micromass model 16F mass spectrometer, using electron impact (70 eV) to ionise the sample. Nuclear magnetic resonance spectra were recorded on a 60 MHz Perkin-Elmer-Hitachi model R-600 spectrometer. Ultraviolet and visible spectra were recorded on a Perkin-Elmer model

552S spectrophotometer. Solutions of drug were kept in the dark whenever possible.

2.1.2 Periodate Oxidation Method

2.1.2a Daunorubicin-bovine serum albumin conjugate

This was prepared essentially as described by Hurwitz et al. (1975) as follows. Daunorubicin hydrochloride (20 mg, i.e. 120 mg Cerubidin) was dissolved in P.B.S. pH 1.4 (1.0 ml), and sodium periodate solution (0.6 ml, 0.1M) was added. The mixture was placed in the dark for one hour with occasional agitation, then glycerol solution (0.1 ml, 1.0M) was added to reduce excess periodate. Bovine serum albumin (30 mg) in potassium carbonate buffer (1.0 ml, 0.15M, pH 9.5) was added and the reaction mixture stored in darkness for two hours. The reaction mixture was then layered onto a Sephadex G50 (coarse grade) column (1.5 x 30 cm), and eluted with distilled water. Visual observation was used to follow the progress of the conjugate fraction through the column. The red fraction which eluted at the void volume of the column (15 ml as judged by prior elution of dextran blue 2000 polysaccharide) was collected and freeze dried.

This gel chromatographic isolation procedure was used for all of the conjugates described below, except where an alternative procedure is described. The daunorubicin content was derived from the absorbance of an aqueous solution of conjugate at the drug λ_{max} of 495 nm, using a molar extinction coefficient of 11065 cm^{-1}. Yield = 15.2 mg (50%), daunorubicin content = 23.3 µg/mg.

2.1.2b Daunorubicin dextran conjugate

Dextran (200 mg, M.W 10,000) was dissolved in sodium periodate solution (10 ml, 0.06M) and kept overnight at room temperature. The

solution was dialysed through a semi-permeable membrane (Spectrapor 1) for 12 hours against distilled water (1L), and then freeze-dried. Daunorubicin hydrochloride (20 mg, i.e. 120 mg Cerubidin) was added to a solution of the freeze-dried material (23 mg) in P.B.S. pH 1.4 1 ml), and stored in darkness overnight at room temperature. Aqueous freshly prepared sodium borohydride solution (81 mM) was added, and the mixture was placed in a covered water bath for two hours at 37°C. The conjugate was isolated as described in section 2.1.2(a) and the amount of daunorubicin present was measured as described in section 2.1.8(a). Yield = 16.5 mg (51%), Daunorubicin content = 157 μg/mg.

2.1.3 Daunorubicin-bovine serum albumin conjugate (conjugated with succinic anhydride)

Succinic anhydride (200 mg) was added to a solution of bovine serum albumin (500 mg) in P.B.S. pH 1.4 (5 ml), and dissolved by gentle swirling. The pH of the solution was monitored using a pH meter and maintained in the range pH 7-8 by gradual addition of sodium hydroxide solution (2M). The solution was kept dark for two hours at room temperature, then dialysed overnight through a semi-permeable membrane (Visking tubing), against P.B.S. pH 7.4 (approximately 500 ml). On the following day the solution was dialysed for four hours against distilled water and then freeze-dried.

Daunorubicin hydrochloride (10 mg, i.e. 60 mg Cerubidin) and 1-cyclohexyl-3(2-morpholinyl-(4)-ethyl)carbodiimide metho-p-toluene sulphonate (400 mg) was added to an aqueous solution of the freeze-dried material (350 mg in 7 ml), and the mixture was stored in the dark overnight at room temperature. The conjugate was isolated as described in section 2.1.8(a) and the daunorubicin content was measured

as described in section 2.1.8(a). Yield = 49.2 mg (49.6%) Daunorubicin content = 32.77 μg/mg.

2.1.4 Synthesis of macromolecular conjugate linked via the daunorubicin C-13 carbonyl moiety.

2.1.4(a) Daunorubicin-bovine serum albumin conjugate (conjugated with 4-hydrazinobenzoic acid)

Bovine serum albumin (1g) was dissolved in P.B.S. pH 7.4 (10 ml). N-Trifluoroacetyl-hydrazinobenzoic acid (250 mg, synthesized according to method 2.1.9(a)) in dioxan (1 ml) and 1-cyclohexyl-3-(2-morpholinyl-(4)-ethylcarbodiimide metho-p-toluenesulphonate (400 mg) were added. The solution was kept dark for three hours and then adjusted to pH 10.5 with aqueous triethylamine (0.2M). The solution was heated at 60°C for 15 minutes to remove the trifluoroacetyl group, then cooled to room temperature and finally dialysed overnight against 0.1M phosphate buffer pH 7.4, then against distilled water for four hours before freeze-drying. Daunorubicin hydrochloride (5 mg, i.e. 30 mg Cerubidin) in P.B.S. pH 7.4 (1.0 ml) was added to a solution of the freeze-dried product (100 mg) in P.B.S. pH 7.4 (2 ml), and kept overnight in the dark at room temperature. The conjugate was isolated by the method described in section 2.1.2 and the daunorubicin content was determined from the absorbance at the drug λ_{max}, of a solution of the conjugate (see section 2.1.8(a). Yield = 49.2 mg (47.6%) Daunorubicin content = 32.77 μg/mg).

2.1.4(b) Daunorubicin-bovine serum albumin conjugate (conjugated with amino(oxy)acetic acid)

Bovine serum albumin (600 mg) was dissolved in P.B.S. pH 7.4 (10 ml). N-trifluoroacetyl-amino(oxy)acetic acid (150 mg, synthesized

according to method 2.1.9(b), and 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-p-toluene sulphonate (350 mg) was added. The solution was kept in the dark overnight and then adjusted to pH 10.5 with aqueous triethylamine (0.2M). The solution was heated at 60°C for fifteen minutes to remove the trifluoroacetyl group, then cooled to room temperature and, finally, dialysed overnight against 0.1M phosphate buffer pH 7.4, then against distilled water for four hours before freeze-drying. Daunorubicin hydrochloride (5 mg) in P.B.S. pH 7.4 (1 ml) was added to a solution of the freeze-dried product (100 mg) in P.B.S. pH 7.4 (2 ml) and kept overnight in the dark at room temperature. The conjugate was isolated by size exclusion chromatography as described in section 2.1.2 and the daunorubicin concentration was found from the absorbance of a solution of conjugate at the drug λ_{max} (see section 2.1.3(a). Yield = 75.22 (75.6%) Daunorubicin content = 25 μg/mg.)

2.1.5 Attempted synthesis of daunorubicin-dextran conjugate (conjugated with glyoxylic acid).

1,3-Diaminopropyldextran (150 mg, synthesized according to method 2.1.9(d), sodium 2-methoxy-2-hydroxyacetate (synthesized according to method 2.1.9(c) and 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluene sulphonate (130 mg), were dissolved in P.B.S. pH 7.4 (1 ml). The solution was kept at room temperature for six hours. Hydrochloric acid (2M) was added until the acidity of the solution reached pH 2-3, and heated under reflux for thirty minutes. After cooling, the solution was neutralised with sodium hydroxide solution (2M), dialysed against distilled water overnight, and then freeze-dried. Daunorubicin hydrochloride (1.5 mg, i.e. 9 mg Cerubidin)

in water (0.3 ml) was added to a solution of the freeze-dried product (90 mg in 1 ml P.B.S. pH 7.4) and the solution was placed in the dark for six hours at room temperature. Isolation of the product was attempted using gel column chromatography as described in section 2.1.8(a). However, no daunorubicin was eluted in the void volume of the column (15 ml as measured using dextran-blue polysaccharide).

2.1.6 Synthesis of anthracycline conjugates coupled with glutaraldehyde

2.1.6(a) Daunorubicin-bovine serum albumin conjugate

Daunorubicin hydrochloride (8 mg, i.e. 48 mg Cerubidin) and glutaraldehyde solution (0.8 ml, 0.4%) were added to a solution of bovine serum albumin (400 mg) in P.B.S. pH 7.4 (4 ml). The solution was stored in the dark for two hours with occasional agitation and the product was then isolated by gel column chromatography as described in section 2.1.2. The daunorubicin content was found from the absorbance at the drug λ_{max} of a solution of the conjugate (see section 2.1.8(a). Yield = 235 mg (57%) Daunorubicin concentration = 19.5μ g/mg.

Studies subsequently performed to determine the extent of daunorubicin release from this conjugate revealed the presence of more than one bond type (see section 1.3.2). The product was passed through a column of porapak Q resin, which absorbed any free drug present. The product from this step was designated fraction I. Incubation of fraction I in P.B.S. pH 7.4 was accompanied by a further release of bound drug, which was also removed by passage through a porapak Q column. The product from this step was designated fraction II.

2.1.6(b) Daunorubicin-bovine serum albumin conjugate (Fraction I)

Daunorubicin-bovine serum albumin conjugate (167 mg) prepared as described in section 2.1.6(a) and isolated by gel column chromatography,

was layered onto a Porapak Q column (0.5 x 5cm) eluted with distilled water. A red fraction that eluted was collected and freeze-dried. The amount of daunorubicin present in the product was measured by visible absorbance and fluorescence methods as described in sections 2.1.8(a) and 2.1.8(b).

Yield = 81 mg (48.3%) Daunorubicin content = 3.6 μg/mg.

2.1.6(c) Daunorubicin-bovine serum albumin conjugate (Fraction II)

A solution of daunorubicin-bovine serum albumin conjugate (containing 600 mg bovine serum albumin and synthesised according to method 2.1.6(a))in P.B.S. pH 7.4 (4 ml) was isolated by gel chromatography as described in section 2.1.2. One half of the solution (approximately) was kept dark overnight at room temperature, then layered onto a Porapak Q column (0.5 x 5 cm), eluted with water and freeze dried.

Yield 303 mg (100%) Daunorubicin content = 3.13 μg/mg.

2.1.6(d) Daunorubicin-horse spleen ferritin conjugate

Daunorubicin hydrochloride (2.5 mg, i.e. 15 mg Cerubidin) in P.B.S. pH 7.4 (0.5 ml) was added to ferritin (30 mg) and glutaraldehyde solution (0.2 ml, 0.4%). The mixture was kept dark for fifteen minutes at room temperature. The conjugate was isolated as described in section 2.1.2(a). The daunorubicin concentration was found from the absorbance of a solution of the conjugate at drug λ_{max} after reduction of the ferritin iron core (see section 2.1.8(c)).

Yield = 11.1 mg (35%) Daunorubicin concentration = 61 μg/mg.

2.1.6(e) Daunorubicin-horse spleen apoferritin conjugate

Daunorubicin hydrochloride (8 mg, i.e. 48 mg Cerubidin) and glutaraldehyde solution (0.4 ml, 0.4%) were added to a solution of

horse spleen apoferritin (50 mg) in water (3 ml). The solution was placed in a covered water bath for one hour at 37°C with occasional agitation. The conjugate was isolated as described in section 2.1.2(a) and the daunorubicin content was measured as described in section 2.1.8(a). Yield = 20 mg (39%) Daunorubicin content = 20.5 µg/mg.

2.1.6(f) Daunorubicin-histone F_1 conjugate

Daunorubicin hydrochloride (1.5 mg, i.e. 9 mg Cerubidin) in P.B.S. pH 1.4 and glutaraldehyde solution (0.2 ml, 0.4%) were added to a solution of histone F_1 (25 mg) in P.B.S. (1 ml). The solution was stored in the dark for fifteen minutes at room temperature with occasional agitation. The conjugate was isolated as described in section 2.1.2(a) and the daunorubicin content was measured by the method described in section 2.1.8(a).
Yield = 18.8 mg (74%) Daunorubicin content = 19 µg/mg .

2.1.6(g) Daunorubicin-1,3-diaminopropyldextran conjugate (conjugated with glutaraldehyde)

Daunorubicin hydrochloride (3 mg, i.e. 18 mg Cerubidin) and glutaraldehyde solution (0.8 ml, 0.4%) were added to 1,3-diaminopropyl-dextran (100 mg synthesized according to method 2.1.9(d))in P.B.S. pH 7.4. The solution was stored in the dark for fifteen minutes at room temperature with occasional agitation, and was then isolated as described in section 2.1.2(a). The daunorubicin content was measured by the method described in section 2.1.8(a).
Yield = 64.7 mg (64%) Daunorubicin content = 8.0 µg/mg.

2.1.6(h) 5-Iminodaunorubicin-bovine serum albumin conjugate

5-Iminodaunorubicin (6 mg) and glutaraldehyde solution (0.4 ml, 0.4%) were added to a solution of bovine serum albumin (200 mg) in P.B.S. pH 7.4 (4 ml). The mixture was stored in the dark for one hour at room temperature with occasional agitation. The conjugate was isolated as described in section 2.1.2(a) and the drug content was measured as described by method 2.1.8(a).
Yield = 112 mg (54%) Drug content = 28.8 μg/mg.

2.1.6(j) Doxorubicin-bovine serum albumin conjugate and 4-demethoxy-daunorubicin-bovine serum albumin conjugate

Doxorubicin hydrochloride (6 mg), and glutaraldehyde solution (0.3 ml, 0.4%), were added to a solution of bovine serum albumin (160 mg) in P.B.S. pH 7.4 (6.6 ml). The solution was stored in the dark for one hour at room temperature with occasional agitation. The conjugate was isolated as described in section 2.1.2(a), and the drug content was measured as described in section 2.1.8(a). This method was also used to prepare 4-demethoxydaunorubicin-bovine serum albumin conjugate.
Yield = 92 mg (57%) Drug content = 8.95 μg/mg.

2.1.6(k) Various anthracycline-bovine serum albumin conjugates

This method described in section 2.1.6(i) was used for the synthesis of all anthracycline-bovine serum albumin conjugates listed in Table 3.7.

2.1.6(l) Glutarated bovine serum albumin

Glutaraldehyde solution (0.4%, 1 ml) was added to a solution of bovine serum albumin (1g) in P.B.S. pH 7.0 (5 ml). The solution was stored in the dark for fifteen minutes at room temperature, and

the product was isolated as described in section 2.1.2(a).

Yield = 609.6 mg (61%).

2.1.7 Daunorubicin-horse spleen ferritin core complex

Daunorubicin hydrochloride (2.5 mg, i.e. 15 mg Cerubidin) ferrous sulphate (6 mg), sodium thiosulphate (90 mg), potassium iodate (27 mg) and apoferritin (15 mg in 0.6 ml water) were dissolved in phosphate buffer pH 7.4 (2 ml). The solution was incubated in the dark at room temperature for 15 minutes with occasional agitation. The conjugate was isolated as described in section 2.1.2(a). The daunorubicin content was measured as described in section 2.1.8(c).

Yield = 10.7 mg (62.5%) Daunorubicin content = 121.5 μg/mg.

2.1.8 Determination of the drug content of anthracycline conjugates

2.1.8(a) Spectrophotometric determination

Anthracycline conjugate (approximately 5 mg) was accurately weighed on a Gallenkamp analytical microbalance and placed in a glass vial. The sample was dissolved in water (2 ml) and the absorbance of the solution was measured at the drug λ_{max} using a dual beam spectrophotometer (Perkin-Elmer 552S), and water as reference. The drug concentration was calculated from the appropriate extinction coefficient given below.

Drug	max	Extinction coefficient (M^{-1} cm^{-1})	Reference
Daunorubicin	495	11065	Bernard et al., 1969
*Doxorubicin	495	12121	Arcamone et al., 1969b
4-Demethoxydaunorubicin	485	9200	Zunino et al., 1976
5-Iminodaunorubicin	592	19800	Tong et al., 1979

* Measured in methanol

4-Deoxydaunorubicin, 4-deoxydaunorubicin, and 3',4'-diepidaunorubicin were measured using the appropriate extinction coefficients for daunorubicin and doxorubicin.

2.1.8(b) Spectrofluorimetric determination

(i) Preparation of the calibration curve

Daunorubicin (0.5 ml, 24 µg/ml) in P.B.S. pH 7.4, was placed in a centrifuge tube and made up to 1.0 ml with P.B.S. pH 7.4. Sulphuric acid (2M, 1.0 ml) was added, and the assay continued as described below. The procedure was repeated with five different concentrations of daunorubicin. A calibration curve of drug fluorescence against concentration was prepared.

(ii) Assay for daunorubicin

Daunorubicin-bovine serum albumin conjugate (2 mg) was accurately weighed on a Gallenkamp analytical microbalance and placed in a clean centrifuge tube. Sulphuric acid (1.0M, 2.0 ml) was added, and the conjugate was dissolved with gentle swirling. The tube was placed in a heating block at 80°C for ten minutes, then cooled to room temperature. Dichloromethane (2 ml, Fisons AnalaR grade) was added and the contents were thoroughly mixed using a vortex mixer. The tube was sealed and centrifuged at 100 x g for five minutes. The aqueous layer was removed with a Pasteur pipette and discarded. A portion of the dichloromethane layer (0.1 ml) was removed using a precision glass syringe, and placed in a silanised test tube. The tube was placed in a heating block for two minutes to evaporate the solvent. Aqueous ethanol (50%, 4 ml) was added and the mixture agitated using a vortex mixer to

ensure all the drug residues were dissolved. The fluorescence of the solution was measured in a Perkin-Elmer model 2000 spectrofluorimeter (excitation = 471 nm, emission = 557 nm). The concentration of daunorubicin in the original sample was found by reference to the calibration curve.

2.1.8(c) Measurement of drug content in ferritin conjugate

Daunorubicin-ferritin conjugate (approximately 2 mg) was accurately weighed on a microanalytical balance, and placed in a clean glass vial. Water (1 ml) was added and the conjugate was dissolved with gentle swirling. 2-Thioacetic acid (1%, 1 ml) in sodium acetate buffer (0.15M, pH 4.6) was added. After ten minutes the absorbance of the solution was measured at 495 nm in a dual beam spectrophotometer (Perkin-Elmer model 552S) against a water blank. The concentration of daunorubicin was calculated assuming an extinction coefficient of 11065 M^{-1} cm^{-1} (Bernard et al., 1969).

2.1.9 Synthesis of linking agents for conjugate preparation

2.1.9(a) N-Trifluoroacetyl hydrazinobenzoic acid

4-Hydrazinobenzoic acid (5g, technical grade) and methanol (500 ml) were placed in an Erlenmeyer flask (1L) and gently heated with stirring until most of the hydrazinobenzoic acid was dissolved. The solution was filtered rapidly into a clean Buchner flask (1L). Water (300 ml) was added and a precipitate of hydrazinobenzoic acid formed. The precipitate was removed by filtration under vacuum and washed with small aliquots of methanol and dried at 40°C overnight *in vacuo*.

A portion of this hydrazinobenzoic acid (3g) was placed in a round-bottomed flask (50 ml) and trifluoroacetic anhydride (10 ml) was

carefully added. The flask was stoppered and kept dark for two days, after which the hydrazinobenzoic acid had completely dissolved. Water (20 ml) was added dropwise, and a white product formed. The product was filtered, washed with water and dried at 40°C for two hours *in vacuo*.

Crude product (500 mg) and methanol (10 ml) were placed in a centrifuge tube and gently heated to dissolve the product. Water (5 ml), was added and the solution was centrifuged for 5 minutes at 1000 x g. The aqueous layer and the top layer of brown solid material were discarded. Methanol (10 ml) was again added and the procedure was repeated. The purified product was dried at 40°C for two hours *in vacuo*.

Yield = 4.8g (76%) m.p. 320-340°C (decomposed).

Elemental analysis C,44.75; H,2.76; N,11.71%. $C_9H_7O_3N_2F_3$ requires C,43.56; H,2.84; N,11.29%.

I.R. (Nujol mull) 3500, 3200, 1775, 1670, 1605, 1240, 1165, 1145, 1015, 940, 890, 720 cm^{-1}.

H^1 P.M.R. (δ from T.M.S. in $CDCl_3$) 7.84 (2H,m), 8.36 (2H,m), 9.100 (1H,S)*, 14.8 (2H,S)*

* Exchangeable with D_2O.

Mass spectrum m/e (5) 207 (1.5), 197 (2), 181 (1.5), 90 (20), 83 (10) 74 (15), 64 (40), 59 (35), 45 (100).

2.1.9(b) N-Trifluoroacetyl-amino(oxy)acetic acid

Amino(oxy)acetic acid hemihydrochloride (1g, 13.9 mmol) was placed in a round-bottomed flask, and trifluoracetic anhydride (3 ml, 21 mmol) was carefully added. The solution was kept for 24 hours at room temperature. Unreacted trifluoracetic anhydride was removed by evaporation under vacuum at 40°C, to yield an oil. Ether (20 ml) was added, and the white crystalline precipitate was removed by filtration

washed with ether and dried under vacuum.

Yield = 886 mg (51%) m.p. = 102-104°C.

I.R. (Nujol mull) 2700, 1780, 1610, 1500, 1480, 1050, 888, 845, 795, 690 cm^{-1}.

Mass spectrum m/e (%) 143 (10), 127 (9), 115 (20), 104 (5), 92 (55), 86 (10), 69 (75), 46 (100).

2.1.9(c) Sodium 2-methoxy-2-hydroxy acetate

Methanol (200 ml), dried by the method of Lund and Bjerrum (1931) was placed in a dry round-bottomed flask (21) fitted with a reflux condenser. Sodium (16g, 0.7M) was added and dissolved without additional warming.

Dichloroacetic acid (16 ml, 0.14M, redistilled) was added dropwise over 15 minutes while the solution continued to boil. The solution was heated over a steam bath for three hours at 90°C, then cooled to room temperature. The solution was neutralised by addition of methanolic hydrochloric acid (6M) and monitored with a pH meter until an acidic pH was reached. Methanol was removed by evaporation under vacuum and the product was recrystallised from methanol/chloroform (1:5 $^{V}/_{V}$).

Yield = 20g (83%) m.p. = 184°C (decomposed).

Elemental analysis: C, 28.25; H, 3.71%. $C_3H_5O_4N$ requires C, 28.11; H, 3.93%.

I.R. (Nujol mull) 3450, 1680, 1320, 1200, 1115, 1055, 946, 917, 810, 787, 746 cm^{-1}.

H^1 N.M.R. (δ from T.M.S. in D_2O) 4.75 (3H,S), 6.15 (1H,S).

Mass spectrum m/e (%) 123 (1), 111 (4), 97 (12), 89 (15), 83 (18), 75 (100), 69 (25), 57 (30).

2.1.9(d) Hydrolysis of sodium 2-methoxy-2-hydroxy acetate

Sodium 2-methoxy-2-hydroxy acetate (300 mg) was dissolved in water (5 ml) and acidified to pH 2 by addition of dilute hydrochloric acid. The solution was heated at 50°C for two hours. Water was removed by evaporation under vacuum. The infrared spectrum of the residue was recorded and compared with the starting material, and a sample of glyoxylic acid (section 3.1).

I.R. (Nujol Mull) 3400, 1730, 1625, 1305, 1260, 1200, 1120, 1070, 1000, 722 cm^{-1}.

2.1.9(e) 1,3-Diaminopropyl dextran

Cyanogen bromide (2g, 18 mmol) was added to a stirred solution of dextran T.40 (2g, Pharmacia Uppsala, Sweden) in water (5 ml). Sufficient sodium hydroxide solution (2M) was added to adjust the mixture to pH 12.5. The solution was continuously monitored throughout the reaction with a pH meter, and maintained in the range pH 11.5-12.5 by further addition of sodium hydroxide solution.

After ten minutes the pH of the solution was readjusted to pH 8 by addition of hydrochloric acid (2M). 3-Aminopropanitrile (1g, 14 mM) was added and the solution was stirred continuously. After sixteen hours the solution was acidified to pH 1 with hydrochloric acid (2M), and dialysed extensively against distilled water before freeze-drying. The freeze-dried residue was suspended in dry ether (200 ml) and lithium aluminium hydride (0.4g, 10.5 mM) was added. The mixture was heated under reflux for two hours. Ether was removed by evaporation under vacuum, and water (100 ml) was slowly added to decompose unreacted lithium aluminium hydride. The aqueous suspension was filtered and freeze-dried. Yield = 430 mg.

2.2 Synthesis of daunorubicin derivatives

2.2.1 Materials

Daunorubicin hydrochloride was purchased as 'Cerubidin' (vials containing 20 mg drug plus 100 mg mannitol) from May and Baker Ltd. (Dagenham, Essex, England), and used without further purification, or as the free base prepared as described in section 2.2.2. Sodium stearate and acetic anhydride were also purchased from May and Baker Ltd.

2.2.2 Preparation of daunorubicin free base

Cerubidin (240 mg containing 40 mg daunorubicin hydrochloride) was dissolved in sodium bicarbonate solution (10 ml, 5%) and extracted with chloroform (3.20 ml). The chloroform extracts were pooled, dried with sodium sulphate, filtered and evaporated to dryness under reduced pressure. The product was dried in a vacuum dessicator over phosphorus pentoxide for seven days.

2.2.3 Synthesis of N-acetyldaunorubicin

Daunorubicin free base (from 40 mg of hydrochloride salt) was dissolved in dry acetone (10 ml). Acetic anhydride (0.5 ml) was added and the solution was stirred for two hours. Acetone was removed by evaporation under vacuum, and the residue was freeze-dried to remove excess acetic anhydride. The crude product was purified by preparative thin layer chromatography (T.L.C.) on Kieselgel 060 plates (1 mm thickness), using chloroform:methanol:acetic acid (90:9:1) as the mobile phase. A red band (Rf = 0.6) was removed, extracted with methanol and the extract evaporated to dryness under reduced pressure.

The residue was purified by high pressure liquid chromatography (H.P.L.C.) using a Lichrosorb RP2 column (0.5-30 cm), and acetonitrile: water (3.5:1.5) as the mobile phase. The mobile phase was eluted at

a rate of 2.0 ml/minute and monitored at 480 nm by a spectrophotometer (Cecil model 273), fitted with a 30 µl flow cell. The residue was dissolved in methanol (2 ml) and injected onto the H.P.L.C. column in aliquots of 0.5 ml. The eluate fraction containing the major product was collected in a clean, dry, round-bottomed flask, and freeze-dried.

Yield = 19 mg (45%) m.p. = 172°C (Lit m.p. 166-177, Yamamoto et al 1972).

H^1N.M.R. (δ from T.M.S. in $CDCl_3$) 1.28 (3H,d), 1.85 (2H,m), 1.95 (3H,S), 2.08 (1H,m), 2.3 (1H,m), 2.4 (3H,S), 2.9 (1H,d), 3.2 (1H,d), 3.5 (3H,S), 3.7 (1H,S), 4.0 (3H,S), 4.2 (3H,m), 5.2 (1H,S), 5.5 (1H,S), 5.9 (1H,d), 7.3 (1H,d), 7.8 (1H,t), 8.0 (1H,d).

Mass spectrum m/e (%) 172 (10), 154 (2), 153 (10), 144 (10), 138 (30), 125 (20), 114 (40), 102 (20), 96 (25), 84 (40), 72 (100).

2.2.4 Synthesis of doxorubicin-14-stearate

Daunorubicin hydrochloride (40 mg, dried over phosphorus pentoxide) was dissolved in a mixture of methanol and dioxan (2 ml) and dry methanol (6 ml). Bromine solution (0.25 ml containing 0.35 ml Br_2 in 10 ml chloroform) was added and the mixture was left to stand for four hours, then filtered and evaporated to dryness under reduced pressure. Acetone (14 ml) and sodium stearate (200 mg) were added and the mixture was heated under reflux for one hour, then filtered and evaporated to dryness under reduced pressure.

The crude product was dissolved in methanol (1.5 ml) and hydrochloric acid (4.5 ml, 0.1M) and extracted several times with chloroform. The extracts were combined, dried with sodium sulphate and evaporated to dryness. The product was recrystallised from chloroform-methanol.

Yield = 18.3 mg (61%).

H^1N.M.R. (δ from T.M.S.) 0.9 (6H,m), 1.2 (24H,S), 1.7-2.7 (18H,S), 2.9-3.2 (2H,m), 3.5 (4H,m), 4.1 (5H,m), 4.4 (1H,S), 5.2 (1H,S), 6.6 (2H,S), 7.0 1H,S), 7.5 (1H,d), 7.8 (1H,t), 8.0 (1H,d), 13.2 (1H,S), 13.7 (1H,S).

I.R. (Nujol Mull) 3480, 1737, 1617, 1580, 1287, 1213, 1170, 1140, 1070, 1030, 990, 810, 720 cm^{-1}.

2.3 Release of drug from daunorubicin-bovine serum albumin conjugate in biological fluids

2.3.1 Materials

Dichloromethane (AnalaR grade) was purchased from Fisons (Loughborough, Leicestershire, UK). Daunorubicin hydrochloride was a gift from Dr. F. Arcamone (Farmitalia, Milan, Italy). The monomorphic Liverpool strain of *Trypanosoma rhodesiense* (Yorke et al., 1929) was used throughout this investigation. Isolation of trypanosomes from infected mice, which is described in section 2.3.3, was carried out by Mr. T.J. Scott-Finigan (N.I.M.R., Mill Hill, London). Preparation of P.B.S. pH 7.4 citrate saline and Krebs glucose solution are described in Appendix I.

2.3.2 Animals

Murine plasma was prepared from male Swiss mice (25-30g) fed on diet 41B (Pilsbury's, Birmingham). Trypanosomes were isolated from infected Parkes mice fed on diet 41B.

2.3.3 Preparation of biological fluids

2.3.3(a) Murine plasma

Swiss mice (25-30g) were killed by cervical dislocation. Blood (0.5 ml) was removed from each mouse by cardiac puncture and placed in a heparinised centrifuge tube. The tube was centrifuged at 1000 x g for three minutes. The plasma layer was removed with a Pasteur pipette and transferred to a glass vial.

2.3.3(b) Trypanosome homogenate

Mice were infected with *Trypanosoma rhodesiense* (see section 2.3.1 and 2.6.3) and the parasitaemia was allowed to proceed until the infection exceeded $10^6 - 10^7$ parasites, ml^{-1}. Blood from ether anaesthetised mice was removed by cardiac puncture and diluted with citrate saline. The blood was centrifuged at 600 x g for ten minutes. The trypanosome layer was removed, resuspended in Kreb's glucose solution and centrifuged at 600 x g for five minutes to give a pellet of trypanosomal material. Water (1 ml) was added to the trypanosomal pellet (1 ml) and the mixture was homogenised in a glass mortar with a teflon pestle for five minutes.

2.3.4 Preparation of calibration curves for daunorubicin in P.B.S. pH 7.4 and Murine Plasma

Daunorubicin hydrochloride (50 µl, 10 µg ml^{-1}) in P.B.S. pH 7.4 was placed in a silanised centrifuge tube. Murine plasma (50 µl) and P.B.S. pH 7.4 (0.9 ml) were added to give a volume of 1.0 ml. Sodium bicarbonate solution (100 µl, 5%) and dichloromethane (1.0 ml) were added and the components were thoroughly mixed using a vortex mixer. The tube was centrifuged at 1400 x g for five minutes. The

aqueous layer was removed and discarded and an aliquot of the dichloromethane layer (0.5 ml) was transferred to a silanised test tube and heated at 45°C under a nitrogen atmosphere until the solvent had evaporated.

Sulphuric acid (4 ml, 0.01M) was added and the mixture agitated using a vortex mixer to ensure all drug residues were dissolved. The fluorescence of the solution was measured in a grating spectrofluorimeter (Perkin-Elmer model 2000) using emission and excitation wavelengths of 552 and 471 nm respectively. The procedure was repeated using differing volumes of daunorubicin solution in the range 0 - 20 µl. The volume of P.B.S. pH 7.4 added was adjusted to give a total volume of 1.0 ml. A graph of fluorescence against drug concentration was prepared (section 3.2). The procedure was also used to prepare a calibration curve of daunorubicin in P.B.S. pH 7.4. For this curve, P.B.S. pH 7.4 (0.95 ml) was added and murine plasma was excluded.

2.3.5 Release of drug from daunorubicin-bovine serum albumin conjugate in murine plasma and phosphate buffered saline pH 7.4

Daunorubicin-bovine serum albumin conjugate (2 mg) was accurately weighed on an analytical microbalance (Gallenkamp) and placed in a silanised stoppered vial (21 ml). Freshly prepared murine plasma (see section 2.3.3(a)) was added, and the vial was sealed, covered with aluminium foil to exclude light, and placed in a water bath at 37°C. Samples (50 µl) were removed at intervals using a precision microlitre syringe and each placed in a centrifuge tube containing P.B.S. pH 7.4 (0.95 ml). Sodium bicarbonate solution (0.1 ml, 5%), and dichloromethane (1.0 ml) were added. Each tube was capped, the contents thoroughly mixed using a vortex mixer, and then centrifuged at 1000g for five minutes to separate the components. The aqueous layer was removed and

discarded. Part of the dichloromethane layer (0.5 ml) was transferred to a silanised test tube and placed in a heating block at 45°C in a nitrogen atmosphere until the solvent had evaporated. Sulphuric acid (4 ml, 0.01M) was added with thorough mixing to ensure that all drug residues were redissolved. The fluorescence emission was measured using a Perkin-Elmer model 2000 spectrofluorimeter (excitation - 471 nm emission - 557 nm).

2.3.6 Release of drug from daunorubicin-bovine serum albumin conjugate in trypanosomal homogenate

The method used was essentially as described in 2.3.5(a), except for minor changes which are given below.

Daunorubicin-bovine serum albumin conjugate (2 mg) was accurately weighed on an analytical microbalance and placed in a clean, silanised glass vial (2 ml). Trypanosome homogenate (0.3 ml), prepared according to method 2.3.3(b), was added and the vial was sealed, covered with aluminium foil to exclude light, and placed in a water bath at 37°C.

Samples (30 µl) were removed at intervals of time using a microlitre syringe and placed in a centrifuge tube containing phosphate buffered saline (0.97 ml).

Preparation and measurement of the sample continued as described in method 2.3.5(a).

2.4 Determination of the affinity of anthracycline drugs for DNA

2.4.1 Materials

Nogalamycin and 7-con-O-methylnogarol were gifts from Dr. P.F. Wiley (Upjohn Co., Kalamazoo, Michigan, USA). N,N-Dibenzyldaunorubicin was a gift from the National Cancer Institute (Bethesda, Maryland, USA).

5-Iminodaunorubicin was a gift from was a gift from Dr. E.M. Acton (Stamford Research Institute, Palo Alto, California, USA), and daunorubicin was a gift from Dr. F. Arcamone (Farmitalia, Milan, Italy). N-acetyldaunorubicin was synthesised as described in section 2.2. Calf thymus D.N.A. (type I) and agarose (type I) were purchased from the Sigma Chemical Co. (Poole, Dorset, England). PM2-DNA was purchased from Boehringer Mannheim (Lewes, Sussex, England). Tris buffer A and tris buffer B were prepared as described in the Appendix.

2.4.2 Determination of the denaturation temperature (Tm) of DNA in the presence and absence of drug.

2.4.2(a) Preparation of DNA solution

Calf thymus DNA (50 mg, Sigma type I) was placed in a small beaker. Tris buffer A (pH 7.4, 50 ml) was added. The beaker was covered with adhesive film and placed in a refrigerator at 4°C. DNA was dissolved by gentle swirling once a day until solid material was no longer visible.

The concentration of DNA was found from the absorbance of a 1:20 dilution, assuming a molar extinction coefficient of 6600 (Plumbridge and Brownm 1978). The concentration of DNA solution prepared by this method was approximately 2.5×10^{-3}M. Calf thymus DNA solution prepared by this method was used throughout this investigation.

2.4.2(b) Thermal denaturation temperature (Tm) of calf thymus DNA

Water (6 ml) and tris buffer (3 ml) were placed in a silanised volumetric flask (10 ml) and sonicated for fifteen minutes to remove dissolved air. DNA solution was added to give a final concentration of 1.5×10^{-4}M. Tris buffer was added so that the final volume of

buffer was exactly 3.6 ml, and the flask was made up to volume with distilled water.

The solution (2.5 ml) was placed in a quartz U.V. cell fitted with a ground glass stopper. Teflon tape was wrapped around the stopper to provide an airtight seal. The cell was placed in the heating compartment of a Perkin-Elmer model 552S spectrometer fitted with a temperature programmer. The spectrophotometer was set at 260 nm, and the temperature of the cell was raised from 58 to 100°C at a rate of 0.5°C/minute. A chart recorder was used to record changes in the absorbance of the sample as the temperature was raised. Tris buffer (3.6 ml in 10 ml water) was used as the reference solution.

2.4.2(c) Determination of the thermal denaturation temperature (Tm) of calf thymus DNA in the presence of drug

For samples containing drug the method was the same as that used in method 2.4.2(b) except for minor changes which are shown below.

A solution of drug (1×10^{-4}M) in water (10 ml) was prepared. Tris buffer (3 ml), water (4.5 ml) and drug solution (1.5 ml) were placed in a silanised volumetric flask (10 ml) and sonicated for fifteen minutes. The volume of drug solution added was adjusted so that the final concentration was exactly 1.5×10^{-5}M. DNA solution was added to give a final concentration of 1.5×10^{-4}M when made up to 10 ml. Tris buffer was added to give a total final volume of 3.6 ml. The flask was made up to volume with water and the experiment continued as described in section 2.4.2(b).

2.4.3 Shift in drug absorption spectrum in the presence of DNA

A solution of drug (1×10^{-4}M) was prepared in water and 5.0 ml was added to each of five silanised volumetric flasks (10 ml). DNA

solution was added to give a range of DNA/drug ratios 0 to 10, and the flasks were made up to volume with tris buffer A (pH 7.4). The spectra of the five solutions were recorded over a range 100 nm either side of the drug λ_{max}, and superimposed.

2.4.4 Effect of pH on the drug absorption spectrum in the presence and absence of DNA

Two solutions were prepared in a silanised volumetric flask (10 ml), the first containing drug (1×10^{-4}M, 5.0 ml) and the second containing drug (1×10^{-4}M, 5.0 ml) and DNA (2.5×10^{-3}M, 0.2 ml) in tris buffer A (pH 7.4). The solutions were made up to volume with tris buffer A (pH 7.4). Two more solutions were prepared containing identical amounts of drug and DNA solution, adjusted to pH 9.4 by addition of sodium hydroxide solution (2M) and made up to volume with distilled water. The spectra of the solutions were recorded over a range 100 nm either side of the drug λ_{max}, and superimposed. The shift in drug λ_{max} which occurred on basification in the presence and absence of DNA was noted.

2.4.5 Spectrofluorimetric titration of drug with DNA

Drug solution (2.5×10^{-6}M, 3.0 ml) in tris buffer A (pH 7.4) was added to two fluorimeter cells and placed in a spectrofluorimeter, together with a reference cell containing tris buffer A (pH 7.4), 3.0 ml). The excitation wavelength was set at the drug λ_{max} and the emission wavelength was found by scanning over a range 30-100 nm above the drug λ_{max}, and the fluorescence emission was recorded. Aliquots of DNA solution (5×10^{-4}M, 20-100 µl) in tris buffer A (pH 7.4) were added to each cell using a precision microlitre syringe. After each addition, the solutions were carefully stirred, and stood for five minutes in the

dark to allow the DNA/drug complex to reach equilibrium; the fluorescence was then recorded. Addition of DNA aliquots continued until no further significant change in fluorescence occurred. The DNA/drug ratios and fluorescence (corrected for volume change) were calculated.

2.4.6 Electrophoretic mobility of covalently closed circular PM2-DNA in the presence of drug

This method was essentially as described by Espejo and Lebowitz (1981). PM2-DNA (50 µg) was dissolved in tris buffer B (pH 7.4, 0.5 ml) and allowed to stand for three days at 4°C. Agarose (0.5g) was dissolved in tris buffer A (pH 7.4, 50 ml) by heating at 100°C for ten minutes, and aliquots of this solution (5 ml) were poured into eight test tubes placed in a waterbath at 60°C. An appropriate volume of drug solution containing 100 µg/ml was added using a precision microlitre syringe, to give a range of final concentration from 0-0.2 µg/ml (0-0.4 µg/ml was used for some drugs). The agarose solutions were carefully poured into electrophoresis tubes (12.5 x 0.58 cm) which had previously been sealed at one end with Nescofilm, and allowed to set (two hours). The tubes were kept dark throughout the experiment to prevent photodecomposition of drug. The upper end of the gel was sliced off and the Nescofilm was replaced by cotton gauze soaked in tris buffer A.

PM2-DNA solution (20 µl) was carefully layered onto the surface of the gels, followed by a solution of bromophenol blue and sucrose (20%, 20 µl). The gels were then kept dark for two hours until the DNA solution had soaked into the gel surface, and placed in a tube electrophoresis cell apparatus. The upper and lower chambers were filled with tris buffer A and subjected to electrophoresis at 50V

until the bromophenol blue indicator had migrated about half the length of the gel (3-4 hours). The gels were extruded and soaked overnight in ethidium bromide solution (0.5 μg/ml). The bands were detected under ultraviolet light and either measured or photographed on Ilford 400 ASA black and white film using an SLR camera fitted with a short focus lens and a red filter.

2.5 Effect of anthracycline drugs on lipid peroxidation in biological systems

2.5.1 Materials

Anthracycline drugs were obtained as previously described in section 2.4.1. NADPH, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company (Poole, Dorset, UK). Thiobarbituric acid was purchased from British Drug Houses (Poole, Dorset, UK), and 1,1',3,3'-tetramethoxypropane was purchased from Aldrich Chemical Company (Gillingham, Dorset, UK). Other chemicals were reagent grade and were used as purchased. NADPH generating solution, Kreb's glucose and citrate saline solutions were prepared as described in the Appendix.

2.5.2 Animals

Swiss male mice (approximately 30g), fed on a normal laboratory diet (diet 41B Pilsbury's, Birmingham), were used in the preparation of liver microsomes. The animals were starved for eighteen hours prior to tissue preparation, but were given drinking water *ad libitum*. Parkes mice (25-30g), fed on normal laboratory diet 41B, were used for experiments involving *Trypanosoma rhodesiense*.

2.5.3 Preparation of mouse liver microsomes

The animals were killed by cervical dislocation and exsanguinated. Each liver was immediately dissected and immersed in a beaker of ice-cold isotonic P.B.S. pH 7.4. All subsequent operations were carried out at 0-4°C. The livers were blotted dry, weighed and homogenised (1g of liver with 2 ml of isotonic P.B.S. pH 7.4) using a Potter-Elveljehm glass mortar homogeniser with a motor driven teflon pestle. The homogenate was centrifuged at 10,000 x g (M.S.E. High Speed 18 refrigerated centrifuge) for thirty minutes in order to sediment cell debris, nuclei, mitochondria and lysosomes. The 10,000 x g supernatant was carefully decanted and centrifuged at 100,000 x g for one hour (Beckmann LE65B ultracentrifuge). The supernatant was discarded and the microsomal pellet resuspended in P.B.S. pH 7.4 (1g of liver in 2 ml) using a vortex mixer.

2.5.4 Preparation of trypanosome suspension

Parkes mice were infected with *Trypanosoma rhodesiense* by intra-peritoneal injection and the parasitaemia was allowed to proceed until the infection exceeded 10^6-10^7 trypanosomes ml^{-1}. The mice were killed by cervical dislocation and blood (0.5 ml) was removed by cardiac puncture and added to citrate saline (1.0 ml). The citrate/blood mixture was centrifuged at 600 x g for ten minutes and the trypanosome layer was resuspended in Kreb's glucose and centrifuged at 600 x g for ten minutes. The pellet was resuspended in culture medium consisting of Kreb's glucose (90%) and inactivated rat serum (10%). The concentration of trypanosome was measured using a haemacytometer and diluted with culture medium to the required concentration.

2.5.5 Preparation of trypanosomal microsomes

Pelleted trypanosomes (2 ml, prepared as described in section 2.5.4) were homogenised at 30,000 r.p.m. for four minutes, followed by 40,000 r.p.m. for one minute, in a Virtis model 60K tissue homogeniser. The homogenate was diluted to 20 ml with sucrose solution (0.25M sucrose, 1 mM E.D.T.A.) and centrifuged at 1000 x g for ten minutes to sediment cell debris. The 1000 x g supernatant was carefully decanted and centrifuged at 105,000 x g for one hour, then at 139,000 x g for a further hour, in a Sorvall 55-34 ultracentrifuge. The 139,000 x g supernatant was discarded and the microsomal pellet was resuspended in phosphate buffer (0.1M, pH 7.4, 2 ml).

2.5.6 Determination of tissue protein content

Microsomal and whole trypanosome protein was assayed by the method of Lowry et al. (1951) as modified by Legget-Bailey (1967). Trypanosomal microsome protein was determined by the method of Whitaker and Granum (1980).

The Lowry method was as follows: Each tissue preparation (10 µl) was diluted 1 in 10 (1 in 50 for certain preparations) with distilled water. Sodium carbonate (1g) was dissolved in sodium hydroxide (0.1M, 50 ml) mixed with copper sulphate (1 ml, 0.5%). Sodium citrate solution was prepared immediately before use from stock solutions of each component (Solution A). An aliquot of solution A (1.0 ml) was added to an aliquot (0.1 ml) of each of the tissue dilutions, and the mixture was allowed to stand for ten minutes at room temperature. Folin-Ciocalteau reagent (0.1 ml, 50%) was added and the solutions were thoroughly mixed, then allowed to stand for thirty minutes, after which the absorbance was determined at 750 nm. A series of standards

containing bovine serum albumin (0-1.0 mg/ml) in distilled water, was used to obtain a calibration curve of absorbance against concentration. Protein content of the tissue preparations was obtained from this graph.

2.5.6(b)

The Whitaker and Granum (1981) method used was as follows: Trypanosomal microsomes (100 µl) were diluted 1 in 20 with dilute sodium hydroxide solution. The absorbance was measured at 235 and 280 nm, and the protein concentration was calculated using the following formula:

$$\text{Protein concentration (mg/ml)} = (A_{235} - A_{280})/2.51$$

2.5.7 Microsomal production of malondialdehyde in the presence of anthracycline drugs

Microsomal suspension (0.1 ml), drug solution (0.3 ml, 0.1 mM) in 0.1M phosphate buffer, and NADPH generating system (0.2 ml) prepared as described in the Appendix, were concentrated at 37°C for thirty minutes (liver microsomes) or one hour (trypanosomal microsomes). The incubation was terminated by addition of trichloroacetic acid (50 mM, 0.3 ml in 50 mM sodium hydroxide). The solution was heated at 90°C for fifteen minutes, cooled to room temperature, then centrifuged in a single speed haematocrit centrifuge (Hawksley, Sussex, England) to remove suspended particles. The absorbance was recorded over the range 580-500 nm and the absorbance at 532 nm was noted. The concentration of malondialdehyde was calculated using an extinction coefficient for the malondialdehyde-thiobarbituric acid complex of $1.51 \times 10^{3} M^{-1} cm^{-1}$. The result was corrected for drug absorbance at 532 nm.

2.5.8 Measurement of malondialdehyde in trypanosome and liver microsomal fractions from mice treated with anthracycline drugs *in vivo*

2.5.8(a) Preparation of samples

Parkes mice were infected with *Trypanosoma rhodesiense* by intraperitoneal injection as described in section 2.6.3. After twenty-four hours, eight mice were given drug (0.5 ml, 0.6 mg/ml in 70% citrate saline/30% murine blood) by intraperitoneal injection. Control mice were given 0.5 ml of 70% citrate saline/30% murine blood. Four hours later four mice from each group were killed by cervical dislocation. The livers were immediately removed and microsomes were prepared from the liver tissue as described in section 2.5.3(a). Blood was taken by cardiac puncture (0.5 ml/mouse), added to an equal volume of citrate saline and centrifuged at 600 x g for ten minutes. The trypanosome layer was carefully removed using a Pasteur pipette, and the malondialdehyde concentration was measured as described below.

2.5.8(b) Measurement of malondialdehyde

Thiobarbituric acid (0.5 ml, 50 mM in 50 mM sodium hydroxide) and trichloroacetic acid (1.0 ml, 10%) were added to tissue suspension (0.5 ml). The solution was heated at 90°C for fifteen minutes, then cooled to room temperature. The solution was centrifuged for two minutes in a single speed haemotocrit centrifuge (Hawksley, Sussex, England). The absorbance was recorded in the range 500-580 nm and the absorbance at 532 nm was noted.

2.5.9 Measurement of oxygen consumption of trypanosomes in the presence of anthracycline drugs

A trypanosome suspension (4 ml, 1.0×10^6 trypanosomes 1 ml^{-1}, prepared according to method 2.5.4) was placed in an oxygen meter

(Yellow Springs Instruments model 53) at 37°C, and the oxygen consumption was recorded. Aliquots of drug solution (1.0 mM in Kreb's glucose) were added to give concentrations of 0.05 and 0.1 mM. After each addition the rate of oxygen consumption was recorded. After each experiment the suspension was microscopically examined and the mobility of the parasite was noted.

2.5.10 Measurement of oxygen consumption of tissue preparations in the presence of anthracycline drugs

Liver microsomes (0.2 ml), prepared according to method 2.5.3(a)) or trypanosome homogenate (0.2 ml, prepared according to method 2.3.3(b)) and NADPH generating solution (0.5 ml prepared as described in the Appendix). were placed in an oxygen meter (S.E.A. model OM1) at 37°C, and the oxygen consumption was recorded. Aliquots of drug solution (1.0 mM in 0.1M phosphate buffer pH 7.4) were added to give concentrations of 0.05 and 0.10 mM. After each addition the rate of oxygen consumption was recorded.

2.6 Testing of daunorubicin and related compounds for activity against *Trypanosoma rhodesiense*

The work described in this section was carried out by Dr. J. Williamson and Mr. T.J. Scott-Finnigan (N.I.M.R., London, England).

2.6.1 Materials

Kreb's glucose and citrate saline solution were prepared as described in Appendix I. Microtest II plates were purchased from Falcon Plastics (California, USA), and automatic microlitre pipettes were purchased from Gibson Ltd. (Villiers-le-Bel, France).

2.6.2 Animals

Parkes mice (20-25g), fed on laboratory diet 41B (Pilsbury's, Birmingham, England) were used in all experiments.

2.6.3 Passaging of *Trypanosoma rhodesiense*

Trypanosomes prepared as described in section 2.5.4 were suspended in citrate saline solution, and the concentration was adjusted to 40,000 parasites/ml using a haemacytometer. An aliquot (0.5 ml) was injected intraperitoneally into a mouse. Death occurred three days after infection.

2.6.4 Preparation of trypanosomes for *in vitro* and *in vivo* drug assays

Blood (0.7 ml) was removed ffrom an ether-anaesthetised mouse, mixed with citrate saline solution (0.3 ml), placed in a centrifuge tube together with further citrate saline (5 ml), and centrifuged at 600g for ten minutes. The supernatant was removed with a Pasteur pipette, the white trypanosome layer was transferred to a fresh centrifuge tube, made up to 10 ml with Kreb's glucose solution and centrifuged at 600 x g for five minutes. This washing procedure was repeated; the trypanosome pellet was finally resuspended in a convenient volume of Kreb's glucose: rat serum (1:1, pre-warmed to 37°C).

2.6.5 *In vitro* testing of daunorubicin and related compounds against *Trypanosome rhodesiense*

In vitro testing was carried out as previously described (Williamson and Scott-Finnigan, 1978) and was as follows:

Infected blood (1 drop) was added to a solution of inactivated calf serum and Kreb's glucose solution (5 ml, 1:1). The concentration was measured using a haemacytometer and adjusted to 10^7 parasites/ml.

Aliquots (0.18 ml) of inactivated calf serum and Kreb's glucose solution were distributed in a Microtest II plate. Medium (0.2 ml) containing drug (10^{-3}M) was placed in one well and an aliquot (20 µl) of this solution was transferred to a second well containing medium (0.18 ml) using an automatic pipette. The drug was serially diluted in the other wells to give a range of concentrations (10^{-3}-10^{-10}M). After adding trypanosome suspension (20 µl) and incubating for four hours at 37°C, trypanosome numbers and motility were assessed using an inverted microscope (ocular x 10, objective x 40). Infectivity was checked by intraperitoneal injection of the well contents into mice (one mouse per well) which were subsequently examined daily for development of parasitaemia. Injection of untreated control suspensions was invariably fatal within four days. Total abolition of infectivity was concluded if no parasites were detectable over a period of 30 days, blood being considered negative if no trypanosomes were seen in 30 microscope fields (ocular x 8, objective x 40) in a coverslip preparation of a drop of blood. Negative parasitaemia followed by a relapse was considered as a temporary abolition of infectivity.

2.6.6 *In vivo* testing of daunorubicin and related compounds against *Trypanosoma rhodesiense*

Infected mice were injected intraperitoneally (0.5 ml/mouse) with aqueous drug solution. Subsequent parasitaemia was followed daily by microscopic examination of tail blood (objective x 40, ocular x 8) in a coverslip preparation. Absence of trypanosomes in 30 microscope fields for at least 30 days was taken as criterion of cure. Intermediate drug effects ranged from:

1) None (treated mice died as rapidly as controls, without showing any reduction in parasitaemia).

2) Treated mice survived longer than controls without showing clearance of parasites from the blood.

3) Treated mice survived longer than controls and showed temporary clearance of parasites from the blood (relapse).

CHAPTER 3 : RESULTS AND DISCUSSION

3.1 Preparation of daunorubicin conjugates and related compounds

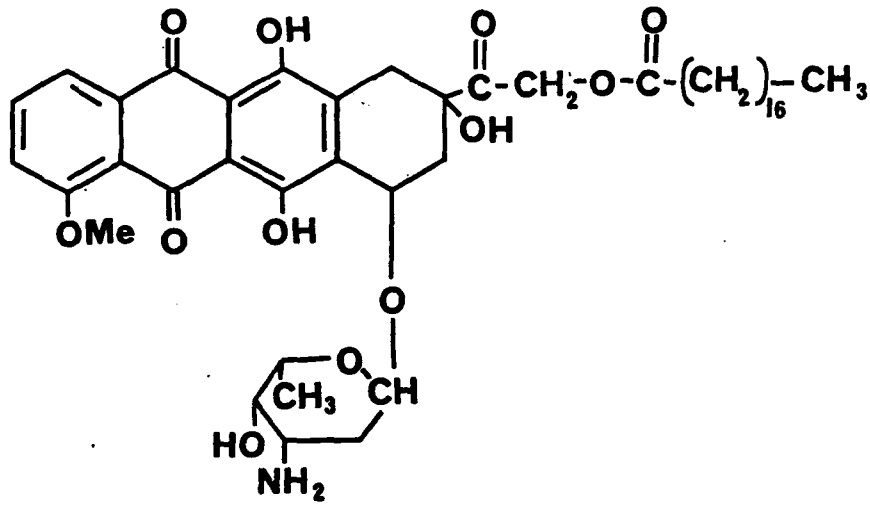
3.1.1 Doxorubicin-14 stearate

Daunorubicin *in vitro* is one of the most active trypanocidal agents known, and abolishes the infectivity of *T.b. rhodesiense* at nanomolar concentrations, but is totally inactive against trypanosomes *in vivo* (Williamson and Scott-Finnigan, 1978). An investigation of this lack of activity showed that, although daunorubicin reaches the parasite in concentrations greatly in excess of that required for activity *in vitro*, its presence is only transitory (Brown et al., 1982a). Since lipophilic pro-drugs are in many cases known to be excreted more slowly than the parent drug (Bundegaard and Hansen, 1981), it was hypothesized that administration of a lipophilic pro-drug derivative would delay clearance of drug from the body and might produce a trypanocidal effect. Also, since the anthracyclines are known to accumulate by diffusion process in tumour cells and possibly in trypanosomes (Skovsgaard, 1977; Brown et al., 1982), a lipophilic pro-drug would also be expected to enhance drug accumulation by the parasite. Enhanced uptake of the lipophilic anthracycline carminomycin by tumour cells has been observed by Kessel (1979). Bachur (1976) has demonstrated a relationship amongst the anthracycline drugs between partition coefficient (lipophilicty) and drug uptake by tumour cells.

The first pro-drug to be prepared was doxorubicin-14 stearate (Figure 3.1), using the method of Arcamone et al. (1974) for the preparation of 14-O-octanoyl and related derivatives of doxorubicin (section 1.4).

Arcamone et al. (1974) demonstrated that doxorubicin-14 esters had high octanol/water partition coefficients and were rapidly hydrolysed to doxorubicin by bloodstream esterases. Lenaz et al. (1974) have suggested that the ester group is hydrolysed intracellularly,

Figure 3.1



thus the stearate group might act as a transmembrane carrier. *T. rhodesiense* shows a unique and rapid uptake of stearic acid (Dixon et al., 1971), hence enhanced uptake and cleavage of doxorubicin-14 stearate might be expected. The parent drug doxorubicin is also a highly active trypanocidal agent *in vitro*, but lacks *in vivo* activity (Williamson and Scott-Finnigan, 1978). The mass spectrum of doxorubicin-14 stearate is presented in Figures3.2 and 3.3. The heaviest fragment, which occurs at m/e 441 is formed after loss of the daunosamine sugar group and the stearate side chain (Williams and Fleming, 1973). The mass fragment at m/e 362 also occurs in the mass spectrum of daunorubicin (Figure 3.3) and its aglycone daunomycinone (Arcamone et al., 1969) and is the 7-deoxyaglycone. In the mass spectrum of daunorubicin-14 stearate, the ions at m/e 380 and m/e 362 might arise from an intramolecular rearrangement (with elimination of carbon dioxide and a hydroxide radical, followed by loss of water (Figure 3.2)). Further identification of doxorubicin-14 stearate is provided by the infrared spectrum. The absorption band for the ester carbonyl group is found at 1736 cm^{-1}. Arcamone et al. (1974a) found this group to lie in the range 1730-1725 cm^{-1} for aliphatic doxorubicin-14-acyl derivatives.

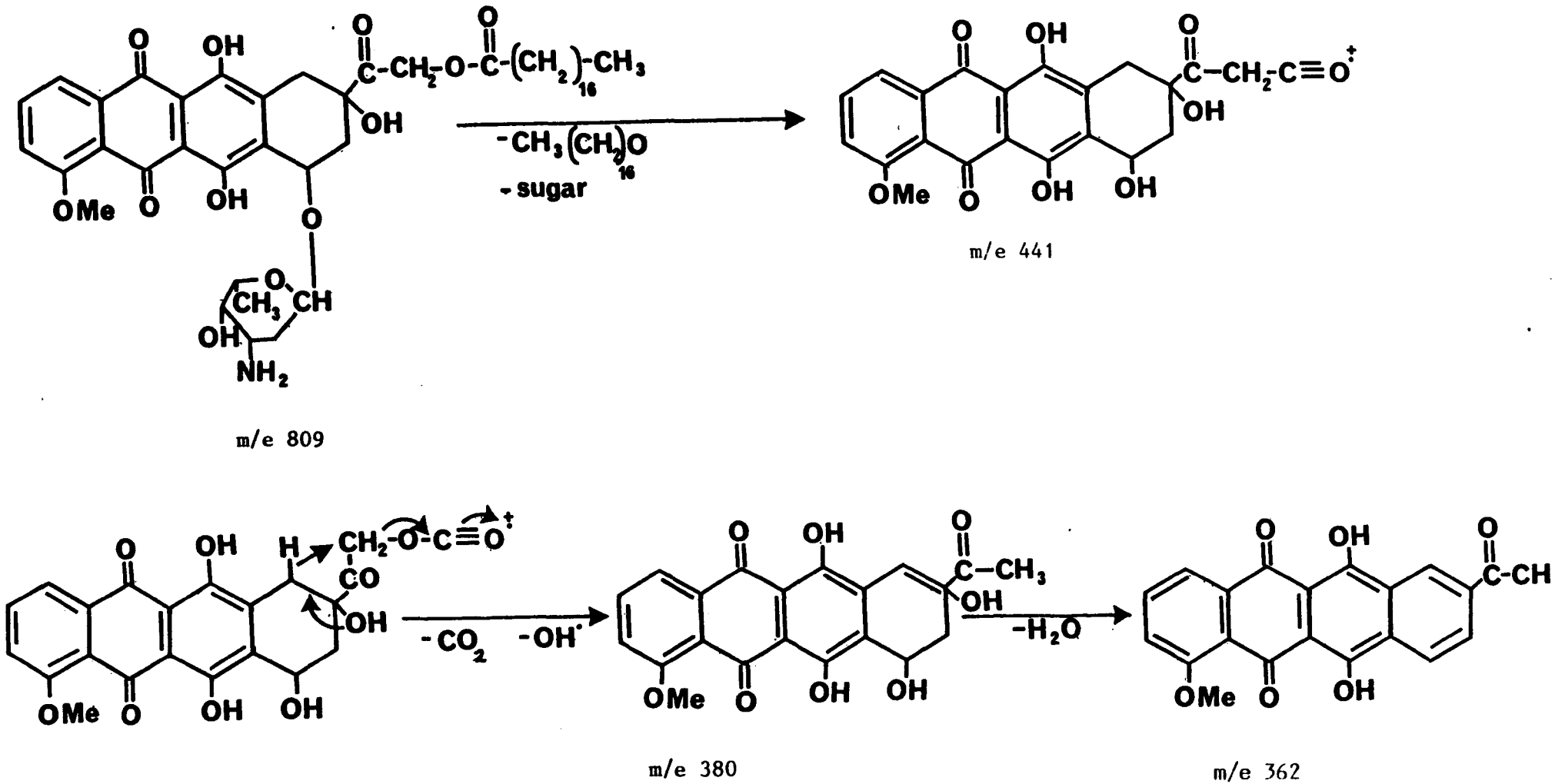


Figure 3.2: Mass spectral fragmentation pattern of doxorubicin-14 stearate

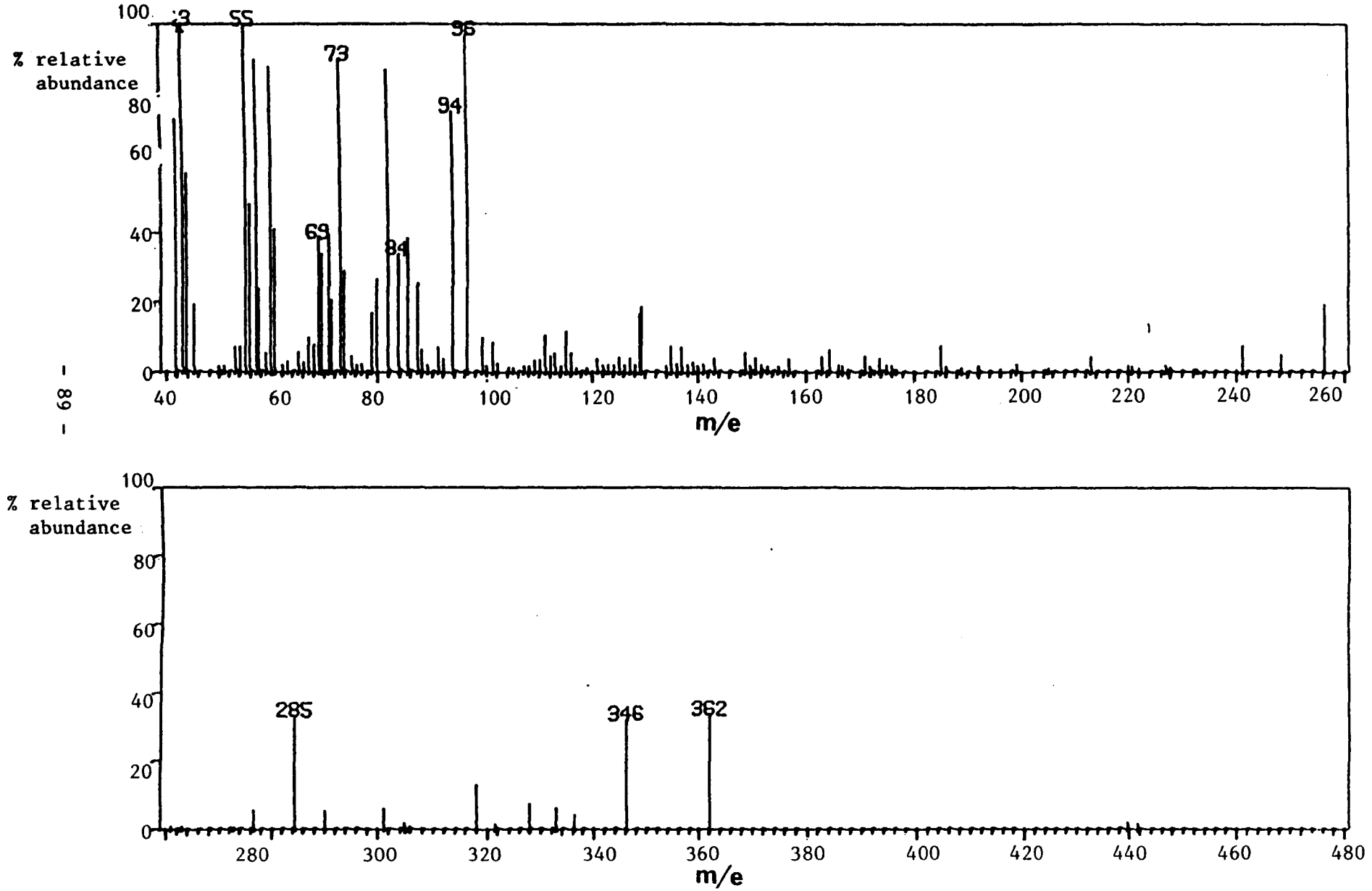


Figure 3.3: Mass spectrum of doxorubicin-14 stearate

In contrast, the C-13 carbonyl peak occurs at 1715 cm^{-1} in the infrared spectrum of daunorubicin and doxorubicin (Smith et al., 1977). Quinone carbonyl groups occur at about 1600 cm^{-1}.

The three aromatic protons of doxorubicin-14 stearate occur in the NMR spectrum at between 7.4 and 8.8 (Figure 3.4) and have an integral height of 5 mm, hence one proton has an integral height of 1.6 mm. The major peak at 1.358 is due to the stearate group and consists of approximately 26 protons. Other protons of the stearate group occur within the multiplet between 0.8 and 2.68. The protons at position C-14 of daunorubicin occur at 2.38 (Figure 3.5), but in this derivative, the protons at C-14 are shifted to approximately 3.48 (Williams and Fleming, 1973) due to the deshielding effect of the ester group and occur as part of the multiplet between 3.4 and 3.68. The details of the other N.M.R. peaks are presented in section 2.25.

Doxorubicin-14 stearate was tested against *Trypanosoma rhodesiense in vitro* (section 3.3) but was found to be inactive. Attention was then turned to the development of daunorubicin macromolecule conjugates as pro-drugs.

A second approach to the development of an effective trypanocide daunorubicin derivative was through the use of macromolecular carriers. Drug-carrier conjugates have been used to sustain release of otherwise rapidly excreted drugs (Bundegaard and Hansen, 1981), and also to alter drug distribution in the body (Gros et al., 1981). Ferritin and albumin were chosen as potential carriers, since they are endocytosed by trypanosomes (Brown et al., 1965; Fairlamb and Bowman, 1977), and therefore intracellular accumulation of bound drug would be expected. Bovine serum albumin was chosen as a macromolecular carrier for the initial studies as it is readily available commercially and possesses high solubility and stability in aqueous solution. The first conjugate

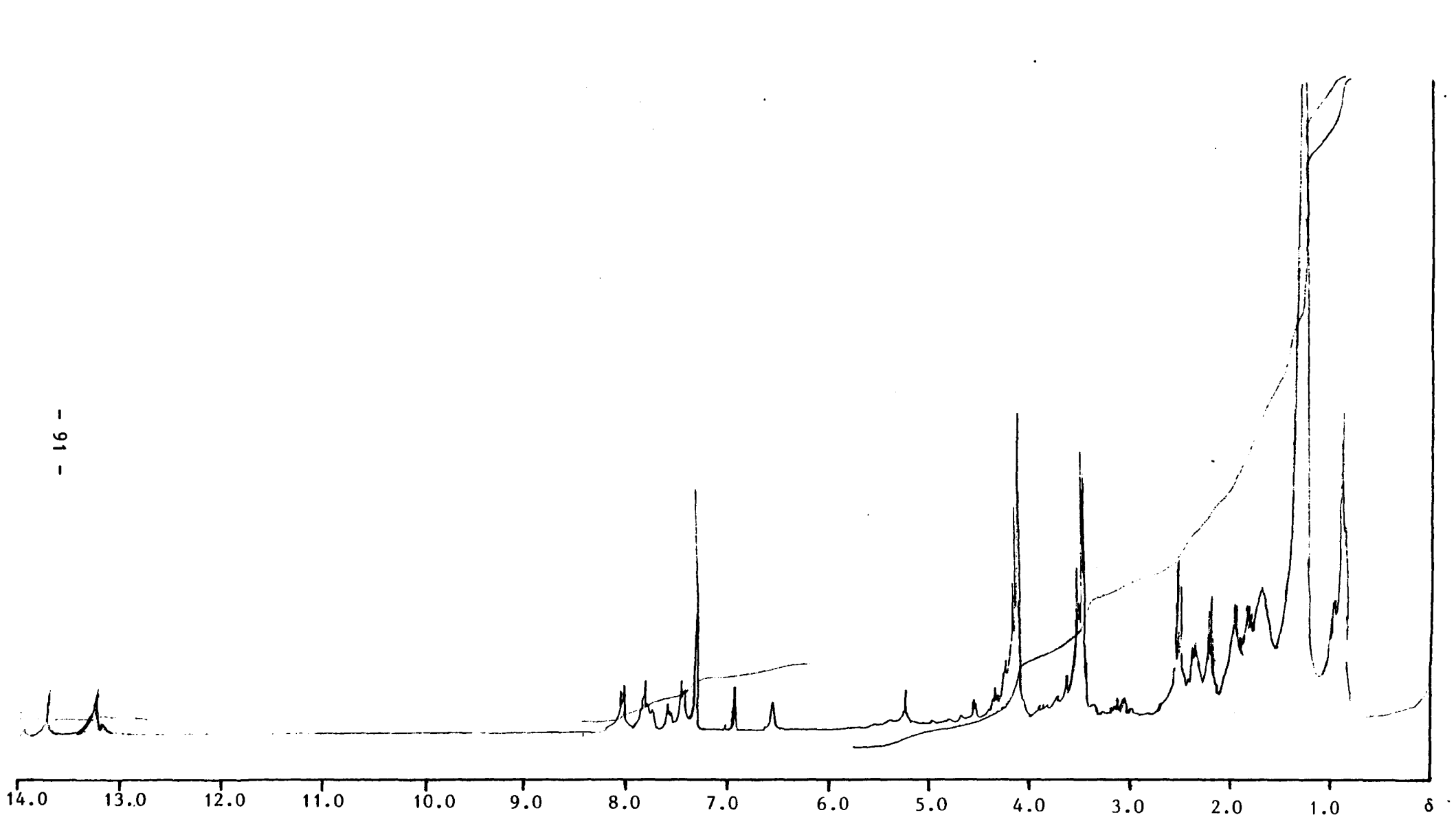


Figure 3.4: 250 MHz NMR spectrum of doxorubicin-14 stearate hydrochloride

was prepared by a method utilising periodate oxidation of the daunosamine sugar group of daunorubicin, followed by reduction of the Schiff's base formed between drug and BSA, using sodium borohydride (Hurwitz et al., 1975). The product was passed through a Sephadex G50 gel filtration column to separate conjugate from free drug, and was then freeze-dried. Since albumin is a carrier protein which possesses non-covalent binding sites for small molecules (Berde et al., 1979) to which daunorubicin could bind, the stability of the conjugate was tested using gel chromatography in 1M sodium chloride and in 2% sodium dodecyl sulphate in 8M urea, to disrupt ionic, hydrophilic and hydrogen bonding. In each test all of the drug was eluted in the void volume of the column, indicating that it was covalently bound to the carrier under these conditions.

This conjugate was tested against *T. rhodesiense in vitro*, but was found to be inactive (Table 3.5). A possible explanation for this lack of activity is that the conjugate linkage is insufficiently labile to provide trypanocidal levels of free daunorubicin (Soudjin, 1977), and the conjugate itself being active. Loss of basic character of the drug, due to cleavage of the $C3^1-C4^1$ bond due to periodate oxidation, may also be a cause of this inactivity since Di Marco and Arcamone (1975) have shown that N-acylation, which reduces the basic character of daunorubicin, is accompanied by a reduction in antitumour efficacy.

A conjugate was then prepared in which daunorubicin was linked to bovine serum albumin using a succinyl spacer group (Figure 3.1.6) linked to drug and protein by amide bonds. This would produce an N-acyl product which is stable since such amide bonds are not readily hydrolysed in biological systems (Soudjin, 1977). Bovine serum albumin was

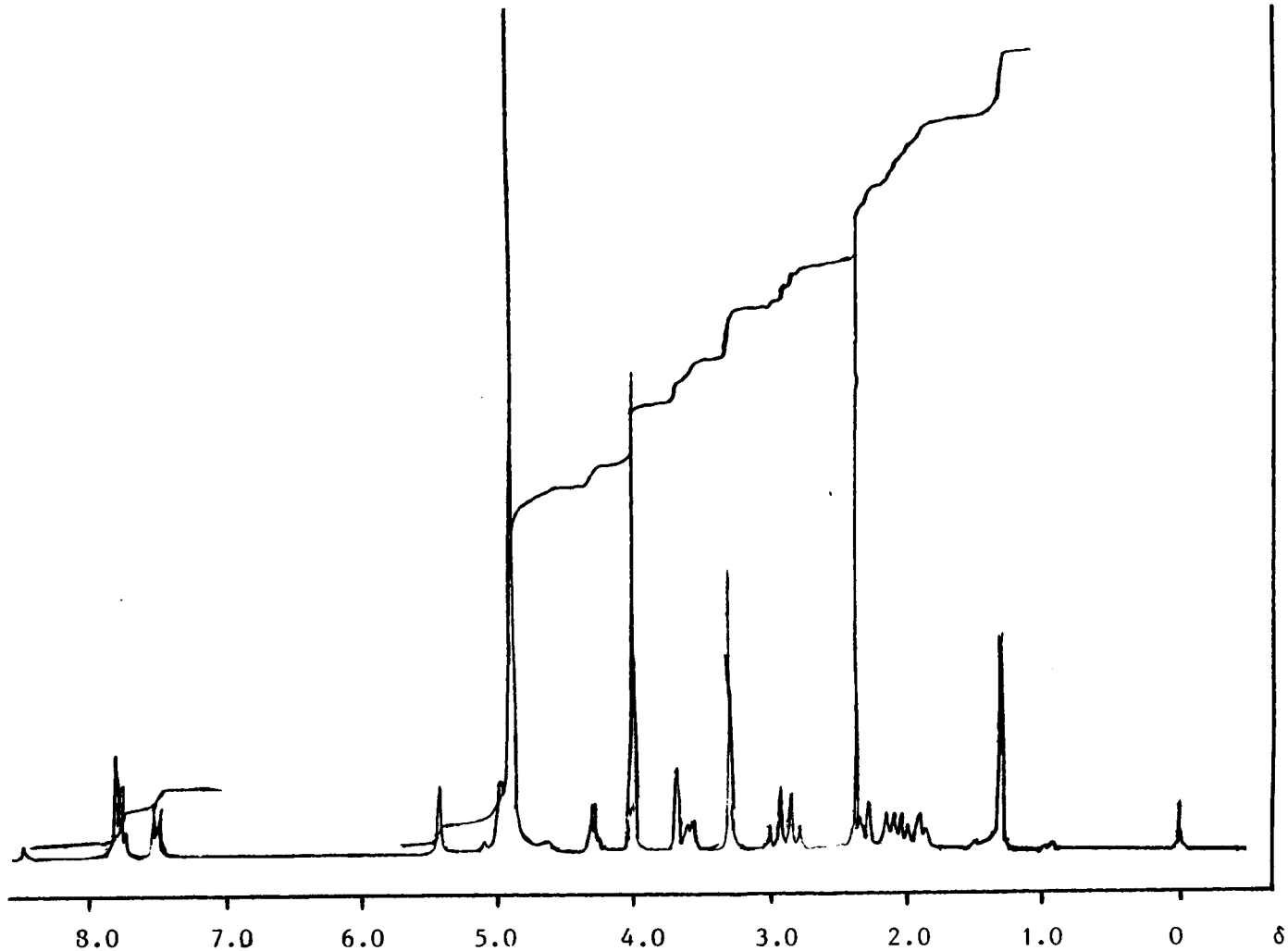


Figure 3.5: 250 MHz spectrum of daunorubicin hydrochloride.

succinylated by reaction with succinic anhydride (Trouet et al., 1982) and dialysed to remove unreacted succinic acid. The succinylated protein was isolated by freeze-drying. Daunorubicin was added to a solution of the derivatised protein and linked using a condensing agent, to form an amide bond.

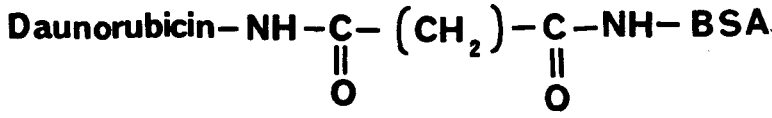


Figure 3.6: Structure of daunorubicin-bovine serum albumin linked via a succinyl spacer group.

The product was stable to 1M sodium chloride and 2% sodium dodecyl sulphate in 8M urea. When tested against *T.b. rhodesiense in vitro* (section 3.4) this conjugate was inactive. Stability of this conjugate was tested in phosphate buffered saline, murine plasma and trypanosomal homogenate, the conjugate was found to be refractory to breakdown in all three media tested (section 3.3). Hence, stable drug conjugate linked via the amino groups are inactive.

In order to provide a conjugate in which daunorubicin would be released unchanged from a labile linkage, a conjugate was prepared using glutaraldehyde as the linking agent. Glutaraldehyde is known to react with amino groups and should form a stable Schiff's base which is susceptible to hydrolysis.

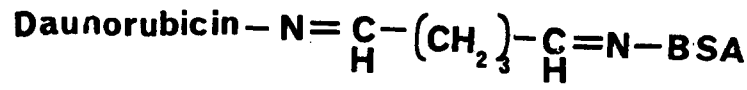


Figure 3.7: Theoretical structure of daunorubicin-bovine serum albumin conjugate linked via a glutaraldehyde spacer group.

A conjugate prepared, using glutaraldehyde, was therefore expected to release daunorubicin as the unchanged drug. Later work (section 3.3) in fact indicated that the structure of the conjugate was more complex than that shown in Figure 3.7. This conjugate was active *in vivo* (Table 3.2.3) as well as *in vitro* (Table 3.3.1) prolonging the lives of mice infected with *Trypanosoma rhodesiense* up to ten days after initial infection. The conjugate also cleared trypanosomes from the bloodstream of the infected animal when given in doses of 7.5 mg/kg of drug and above (Table 3.3.3). However, no cures were obtained.

In order to optimise the efficacy of daunorubicin macromolecule conjugate, two lines of investigation were pursued:

1) Variation of the type of carrier macromolecule in order to identify the characteristics required for optimum uptake of the conjugate by trypanosomes.
2) Variation of the daunorubicin macromolecule linkage and determination of drug release with time so as to identify the characteristics of the linkage required for activity.

The macromolecules used were chosen with regard to their different physical and chemical characteristics. Daunorubicin was linked via glutaraldehyde to ferritin, apoferritin, histone, and dextran derivatised to contain amino groups. These macromolecules provide a range of molecular weights and electrical charge. The characteristics of the resulting conjugates are shown in Table 3.1; all of these conjugates were found to be active *in vivo*.

Macromolecule	Molecular Weight ($\times 10^{-3}$)	Characteristics	Daunorubicin loading (drug/mg protein)	% saturation* of protein	Trypanocidal activity *in vivo*
None					Inactive
Bovine serum albumin	69	Globular Protein (neutral)	18	3.6	Active
Horse spleen ferritin	900	Globular Protein (acidic)	55	3.8	Active
Horse spleen Apoferritin	440	Globular Protein (acidic)	20.5	0.7	Active
Calf Thymus Histone f_1	10	Globular Protein (basic)	19.7	-	Active
Polyamino-dextran	40	Polysaccharide (basic)	8.6	-	Active

* Determined from the molar ratio of drug to protein and expressing this as a percentage of the number of protein amino groups theoretically avaliable for covalent binding.

Table 3.1: Characteristics of daunorubicin-macromolecular conjugates linked via glutaraldehyde

3.1.2 Daunorubicin-ferritin conjugate

Ferritin was of particular interest as a carrier, since Brown et al. (1965) observed *in vitro* uptake of ferritin by *Trypanosoma rhodesiense*. Ferritin contains a core of ferric hydroxide surrounded by twenty-four sub-units of apoferritin and can be prepared from apoferritin and ferrous ion in the presence of an oxidising agent (Crichton, 1973). Since daunorubicin forms a stable complex with iron (Gosalvez, 1977), preparation of daunorubicin-ferritin complex by sequestration of daunorubicin in the ferritin core was attempted. Daunorubicin would be expected to be released from the ferritin when the complex is endocytosed and probably metabolised by trypanosomes (Langreth and Balber, 1975). Oxidation of ferrous sulphate in the presence of apoferritin and daunorubicin produced a conjugate which was stable to 1.0M sodium chloride and 2% sodium dodecyl sulphate in 8M urea, although drug was released after reduction of ferritin with 1% thioacetic acid. This conjugate possessed a very high drug loading (121 µg/mg ferritin), but was inactive *in vivo*. This may be due to the high stability of the daunorubicin iron complex.

3.1.3 Daunorubicin conjugate linked via the C-13 carbonyl group

Although conjugates prepared using glutaraldehyde were active, it is not an ideal linking agent. Due to its bifunctional nature, protein cross-linking can occur which may lead to loss of solubility and, if antibodies are used as the carrier, to loss of specificity (Hurwitz et al., 1975). In addition, since this method requires a primary amine group, daunorubicin derivatives which do not contain such a group cannot be used. In order to circumvent these problems, and to investigate the characteristics of the linkage required for optimum activity, an attempt was made to develop further conjugates by exploiting the carbonyl group

at position C-13 of daunorubicin. The linking agents 1,4-hydrazinobenzoic acid and amino(oxy)acetic acid were chosen as they readily react with carbonyl groups to give a hydrazone and an oxime respectively (Erlanger, 1976). The linking agent was coupled to bovine serum albumin via an activated ester of the acidic function using a carbodiimide to form an amide bond (see Figures 3.8(a) and (b)). Prior to this, the basic moieties of the cross-linking agents were protected by trifluoracetylation since self-condensation of the linking agent would occur. Subsequent to the coupling to protein, trifluoroacetyl groups were removed by mild hydrolysis under basic conditions.

During this procedure, bovine serum albumin was not apparently denatured since no precipitation of material was observed. Incubation of daunorubicin with 1,4-hydrazinobenzoic acid and amino(oxy)acetic acid linked to bovine serum albumin produced conjugates which were unstable (section 3.3) and were not active *in vivo*.

3.1.4 Glyoxylic acid linked daunorubicin conjugate

Glutaraldehyde is known to form at least two types of linkage between protein and drug (section 3.3). The lability of this type of linkage will probably be different from that of a simple Schiff's base linkage, since a glutaraldehyde linkage might rearrange to form an enamine (Sollenberger and Martin, 1968). Simple Schiff's bases are known to be reversible (Sollenberger and Martin, 1968), and a drug-protein conjugate linked by a simple Schiff's base would be expected to release drug rapidly *in vivo* and so might produce a trypanocidal effect. Glyoxylic acid was chosen as a potential linking agent as the carboxyl group may be linked to the carrier molecule and daunorubicin may be linked to the aldehyde group via Schiff's base formation. Before glyoxylic acid can be linked to protein, the aldehyde moiety

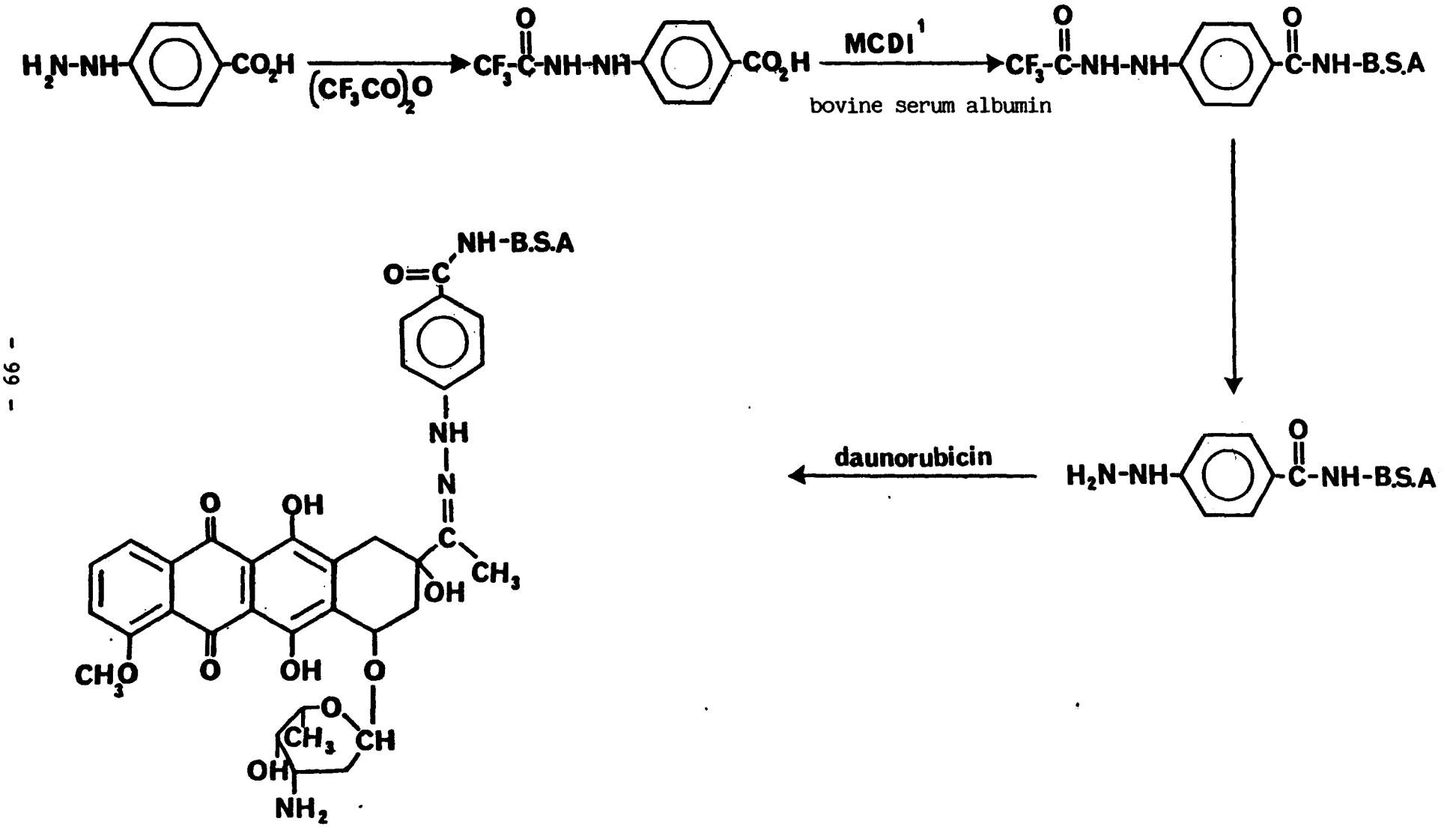


Figure 3.8(a): Preparation of daunorubicin-bovine serum albumin conjugate, linked via: a) 1,4-hydrazinobenzoic acid

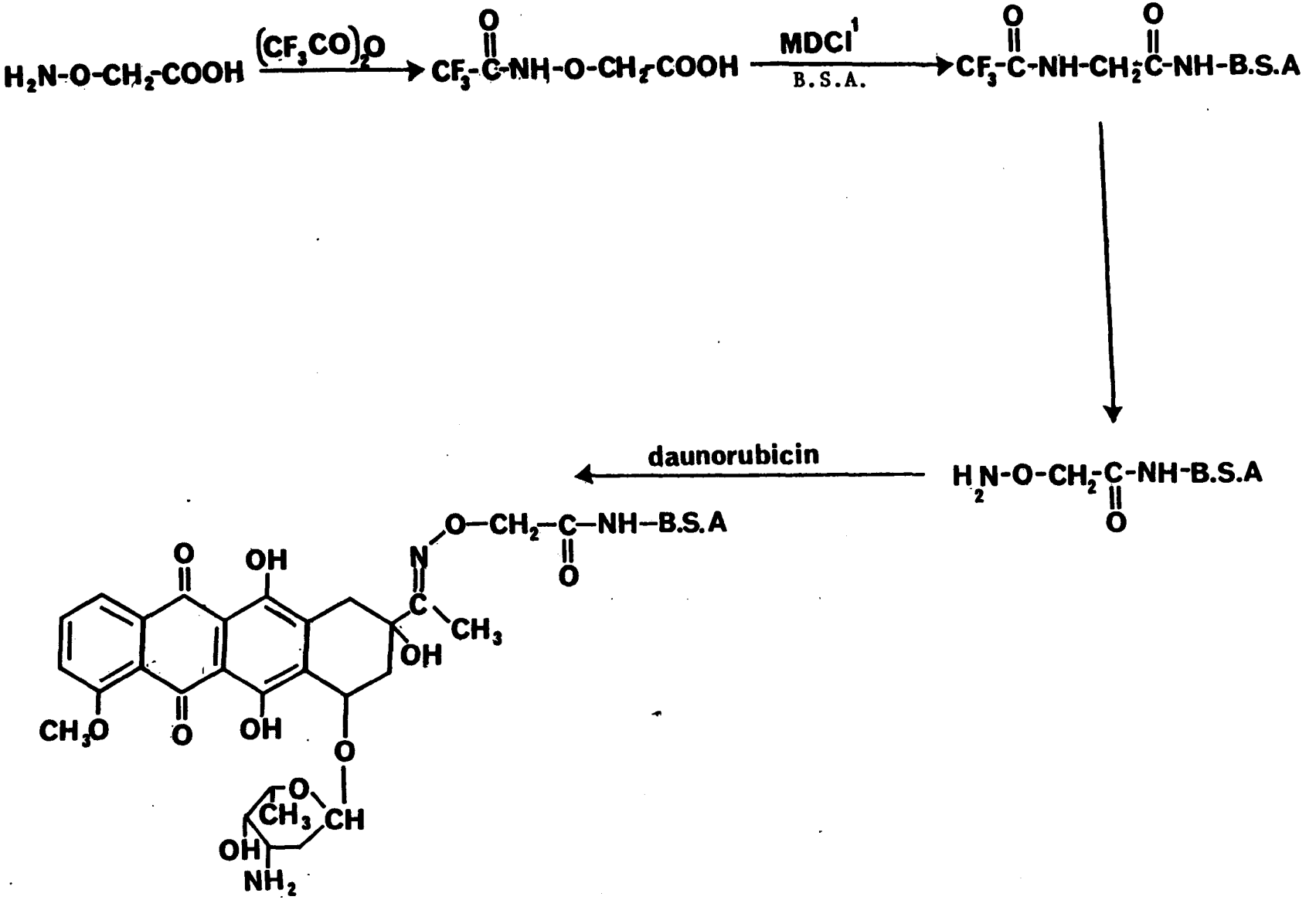


Figure 3.8(b): Preparation of daunorubicin-bovine serum albumin linked via: b) amino(oxy)acetic acid

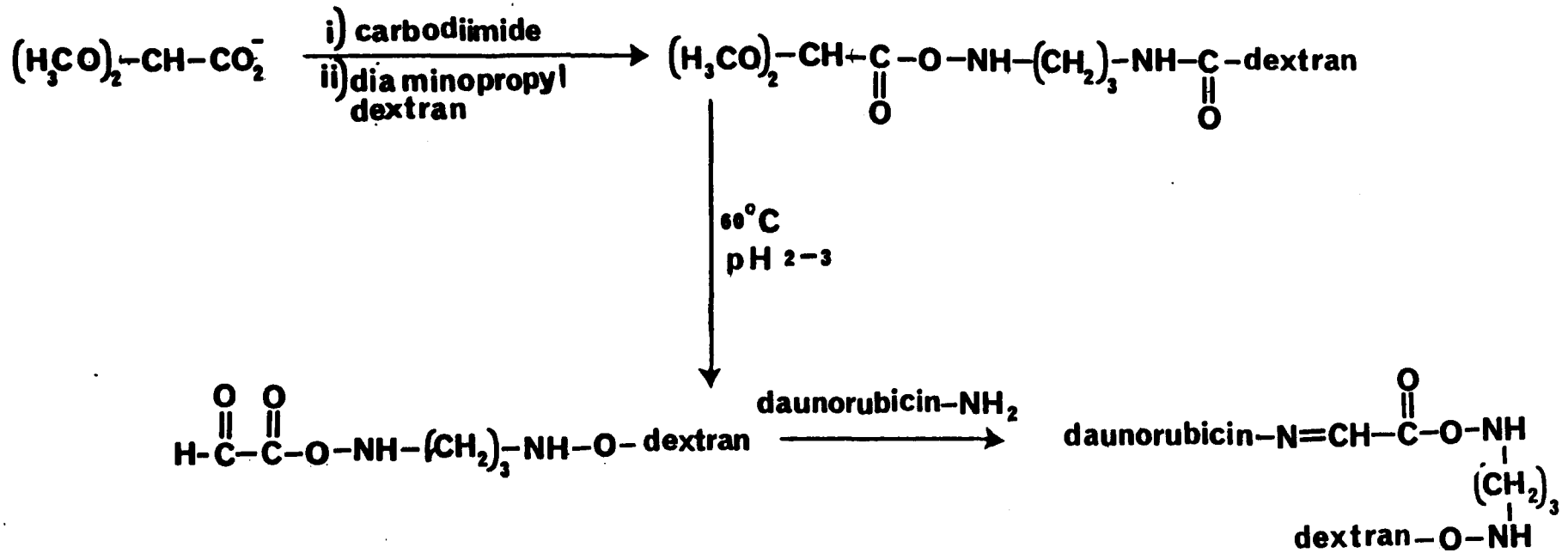


Figure 3.9: Attempted preparation of daunorubicin-dextran conjugate linked via glyoxylic acid

must be protected against nucleophilic attack by the condensing agent. The sodium salt of glyoxylic acid hemiacetal (sodium-2-methoxy-2-hydroxyacetate) was synthesised (section 2.1.9(c)) as these compounds are known to be readily hydrolysed by dilute acid, to give the aldehyde group. Elemental analysis and other data presented in section 2.1.9(c) support the formula given. In order to find the condition necessary to generate glyoxylic acid, a small sample was heated with dilute acid for two hours. The water was removed by evaporation under vacuum and the infrared spectrum of the product was recorded (Figure 3.10b). The spectrum shows marked changes from that of the starting material (Figure 3.10b) and several of the major peaks are characteristic of glyoxylic acid, including aldehyde stretch at 1730 cm^{-1} (Figures 3.1.10b and 3.1.11). Some peaks characteristic of the starting material are still present, indicating that hydrolysis is not complete. Under the conditions required to generate glyoxylic acid, proteins would be denatured and are therefore unsuitable as carriers. Cyanogen bromide activated 1,3-diaminopropyl-dextran was used as the carrier since cyanogen bromide activated conjugates are known to be stable in acid solutions (Axen et al., 1967). Preparation of the conjugate proceeded as described in section 2.1.6(g), for reaction sequence see Figure 3.9. However, a product could not be isolated by Sephadex gel chromatography, presumably due to rapid hydrolysis of the Schiff's base linkage.

3.1.5 Comparison of the absorbance and fluorescence methods for the measurement of daunorubicin in macromolecular conjugates

In accordance with earlier workers (Hurwitz et al., 1975) the drug content of conjugates was found by measurement of absorbance at 495 nm using an extinction coefficient ($E^{1\%}_{1\ cm}$) of 196. This may not give an accurate estimate of the amount of drug bound since it refers to the

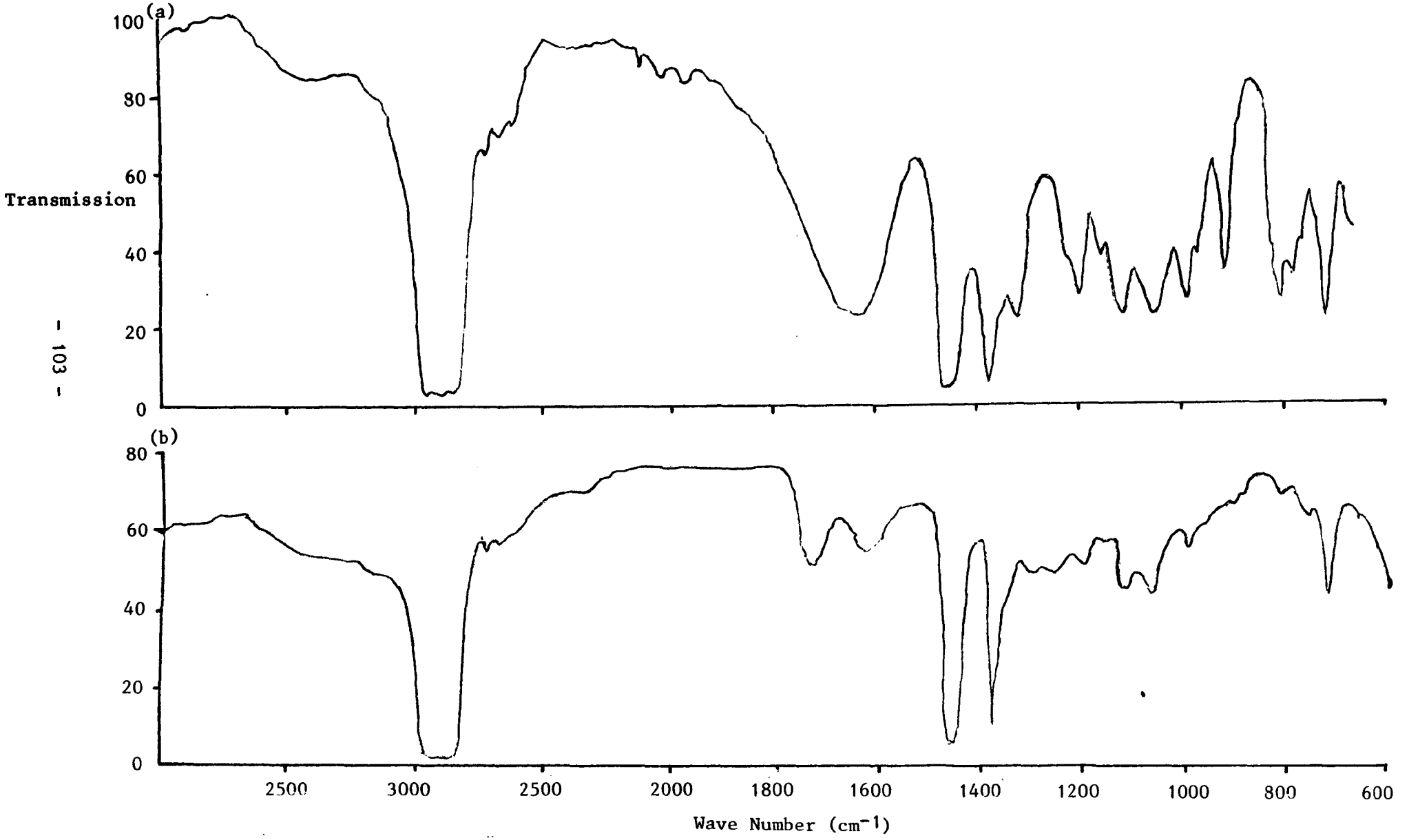


Figure 3.10: Infrared spectrum of (a) sodium 2-hydroxy-2-methoxyacetate: (b) sodium 2-hydroxy-2-methoxyacetate after hydrolysis.

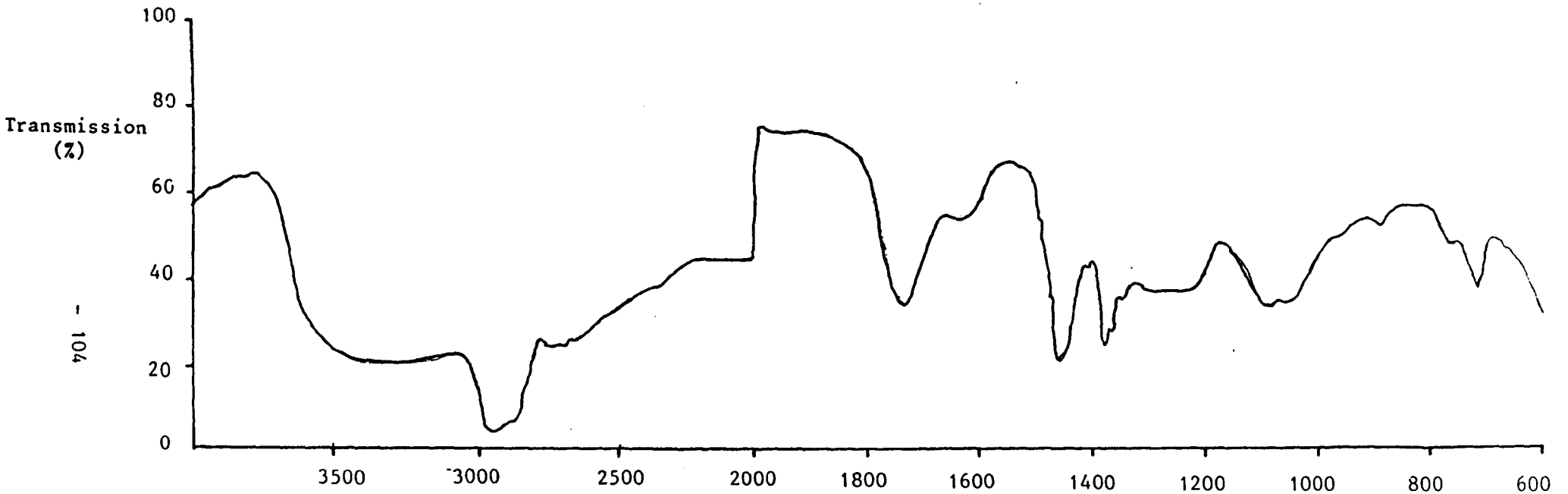


Figure 3.11: Infrared spectrum of glyoxylic acid

free drug. Linkage of drug to a macromolecule may also alter the extinction coefficient. In order to demonstrate whether this method gave a reliable estimate of the amount of drug bound, an independent method of drug estimation was developed (section 2.1.8) based on hydrolysis of drug aglycone from the carrier, followed by fluorimetric determination of the aglycone. A calibration curve prepared using aqueous daunorubicin solution is shown in Figure 3.12. The correlation coefficient was greater than 0.999. A daunorubicin-bovine serum albumin conjugate was analysed by both methods and the results are presented in Table 3.2.

Table 3.2: Comparison of the daunorubicin content of five samples of a BSA conjugate analysed by spectrophotometric and fluorimetric methods

Sample	Weight (mg)	Concentration of daunorubicin ($\mu g\ mg^{-1}$) measured by:	
		Absorbance	Fluorescence
1	8.282	13.7	15.3
2	6.126	13.6	14.6
3	6.912	13.5	15.1
4	10.021	9.4	6.1
5	8.672	13.2	13.9
Mean	=	12.68	13.0
Standard deviation	=	0.83	1.7

Analysis using student's t-test indicated no significant difference between these methods ($p = 0.05$). If Sample 4, which appears to be anomalous, is excluded, the mean become 13.7 and 14.7 for the absorption and fluorimetric methods respectively. Student's t-test also indicated

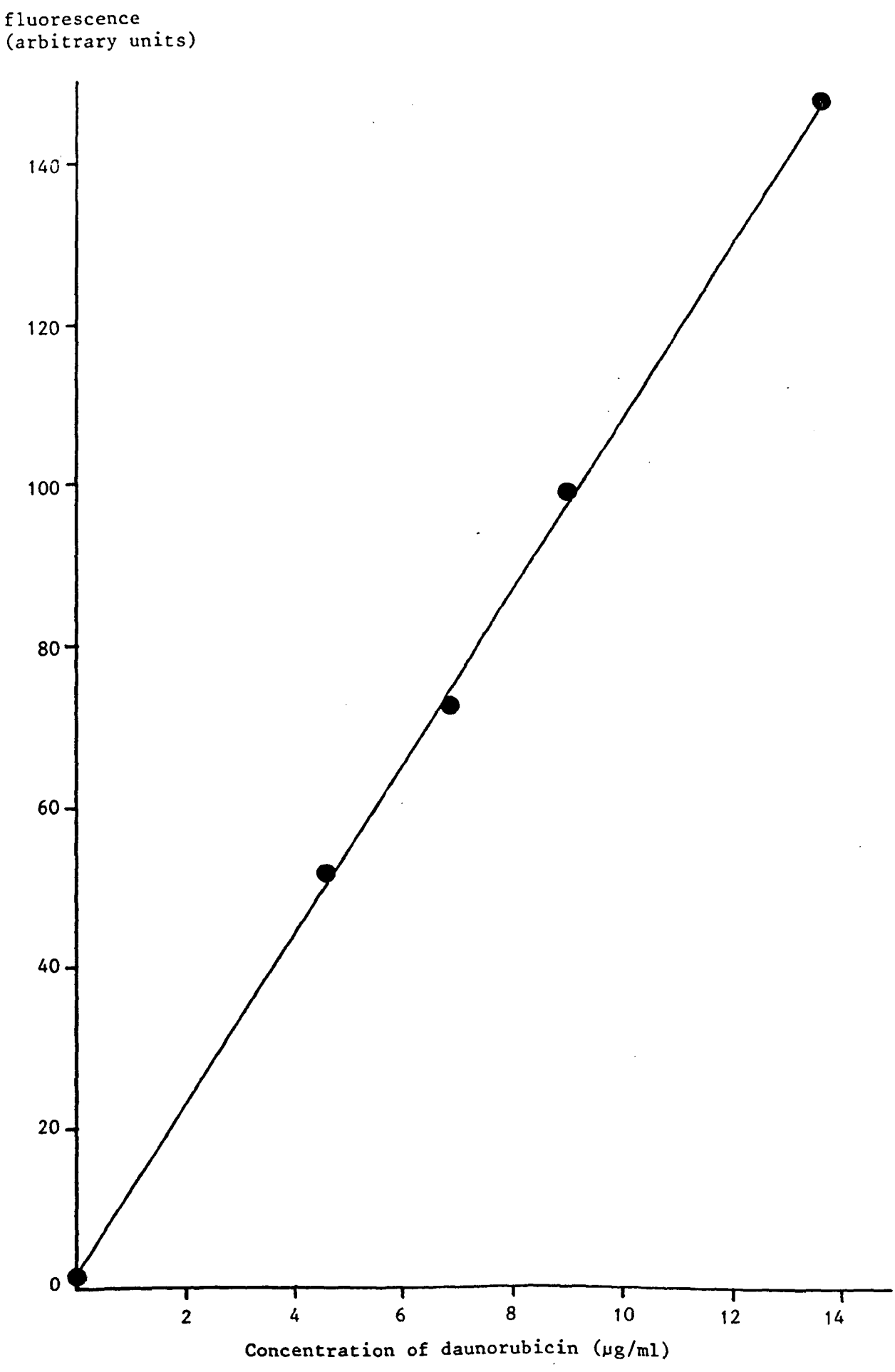


Figure 3.12: Calibration curve for fluorescence assay of daunorubicin

that the difference between the means was not significant. These results show that these methods produce similar results, and that differences in sample homogeneity are greater than differences between the assay methods.

3.1.6 Preparation of N-acetyldaunorubicin

N-Acetyldaunorubicin was prepared by the method of Yamamoto et al. (1972). This derivative is similar in structure to daunorubicin, but was found not to intercalate into DNA, and was used to compare the effect of a non-intercalating daunorubicin derivative with that of daunorubicin. The NMR spectrum (Figure 3.5.1) is identical with that published by Arcamone et al. (1969) except for a peak at 3.58 δ. This peak is also present in the NMR spectrum of daunorubicin (Figure 3.13) and is probably due to water, which does absorb in this region (Williams and Fleming, 1973). The melting point of 172°C is in the middle of the range of 166-177°C reported for N-acetyldaunorubicin by Yamamoto et al. (1972). The mass spectrum of N-acetyldaunorubicin does not contain peaks above m/e 171; however there is a series of peaks present in the mass spectrum which are characteristic of this compound (Figures 3.14 and 3.15).

This chapter has shown the rationale behind developments of daunorubicin derivative and macromolecular conjugates and given some indication of their activity against *T. rhodesiense*. In the following chapter the activity of these conjugates is considered in detail.

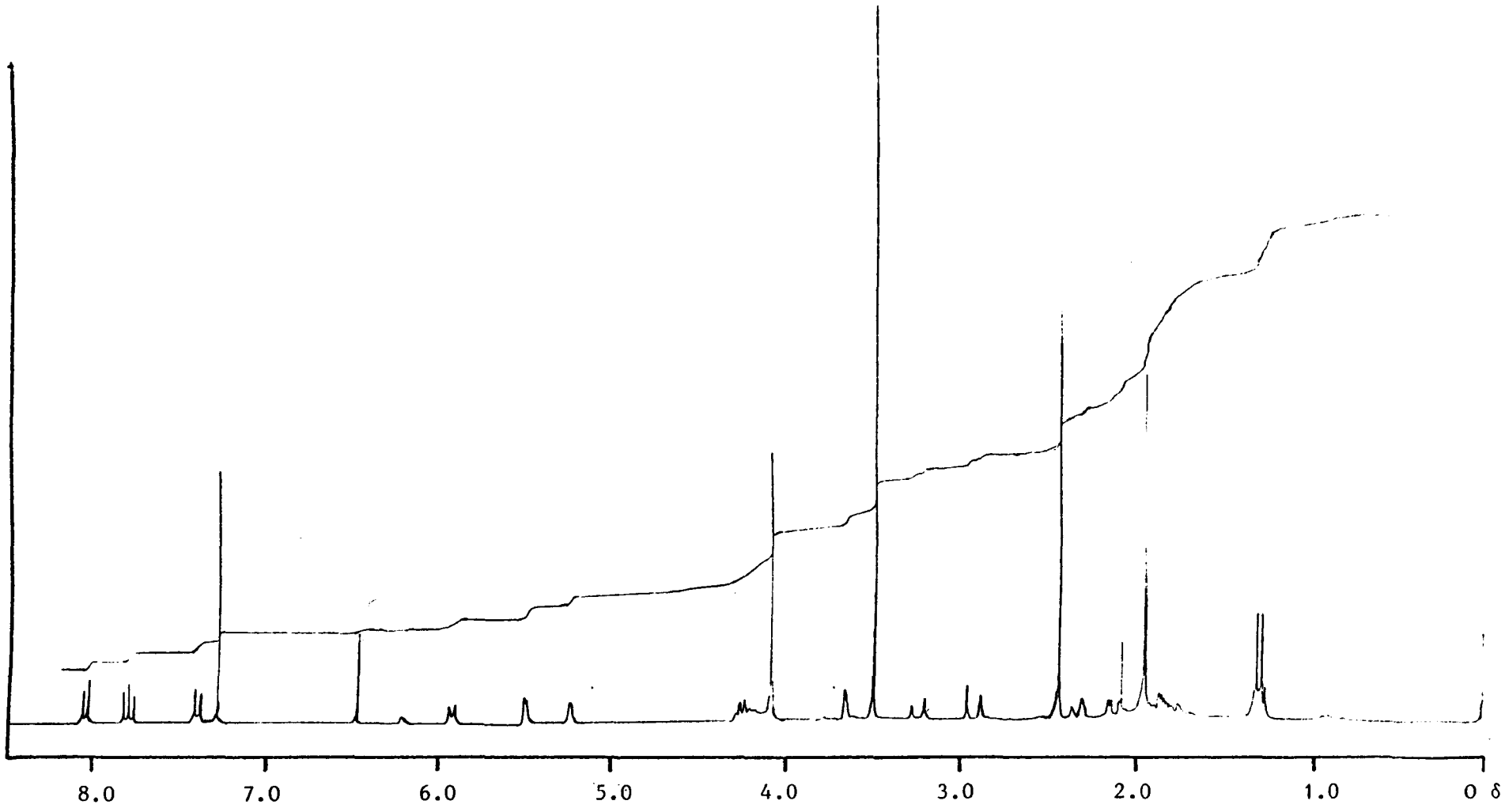


Figure 3.13: 250 MHz NMR spectrum of N-acetyldaunorubicin in $CDCl_3$

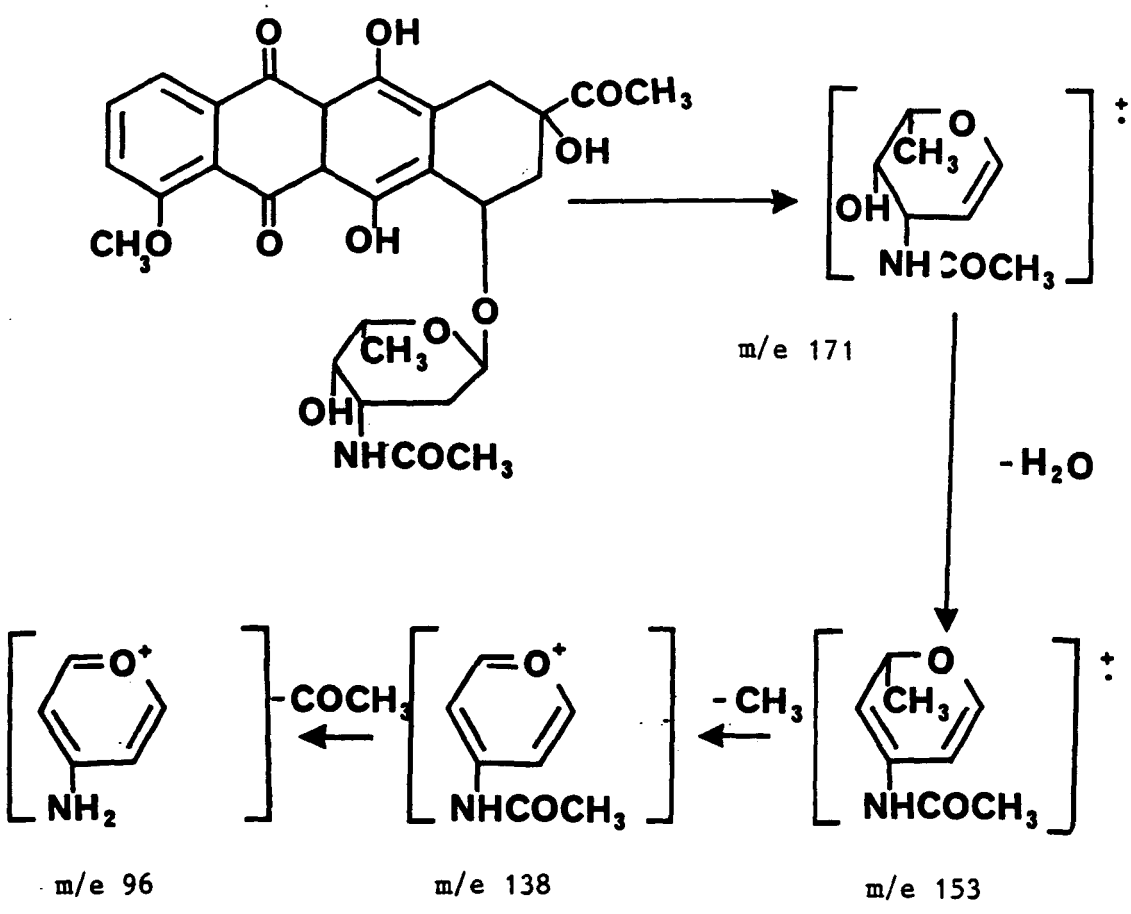


Figure 3.14: Mass spectral fragmentation of N-acetyldaunorubicin

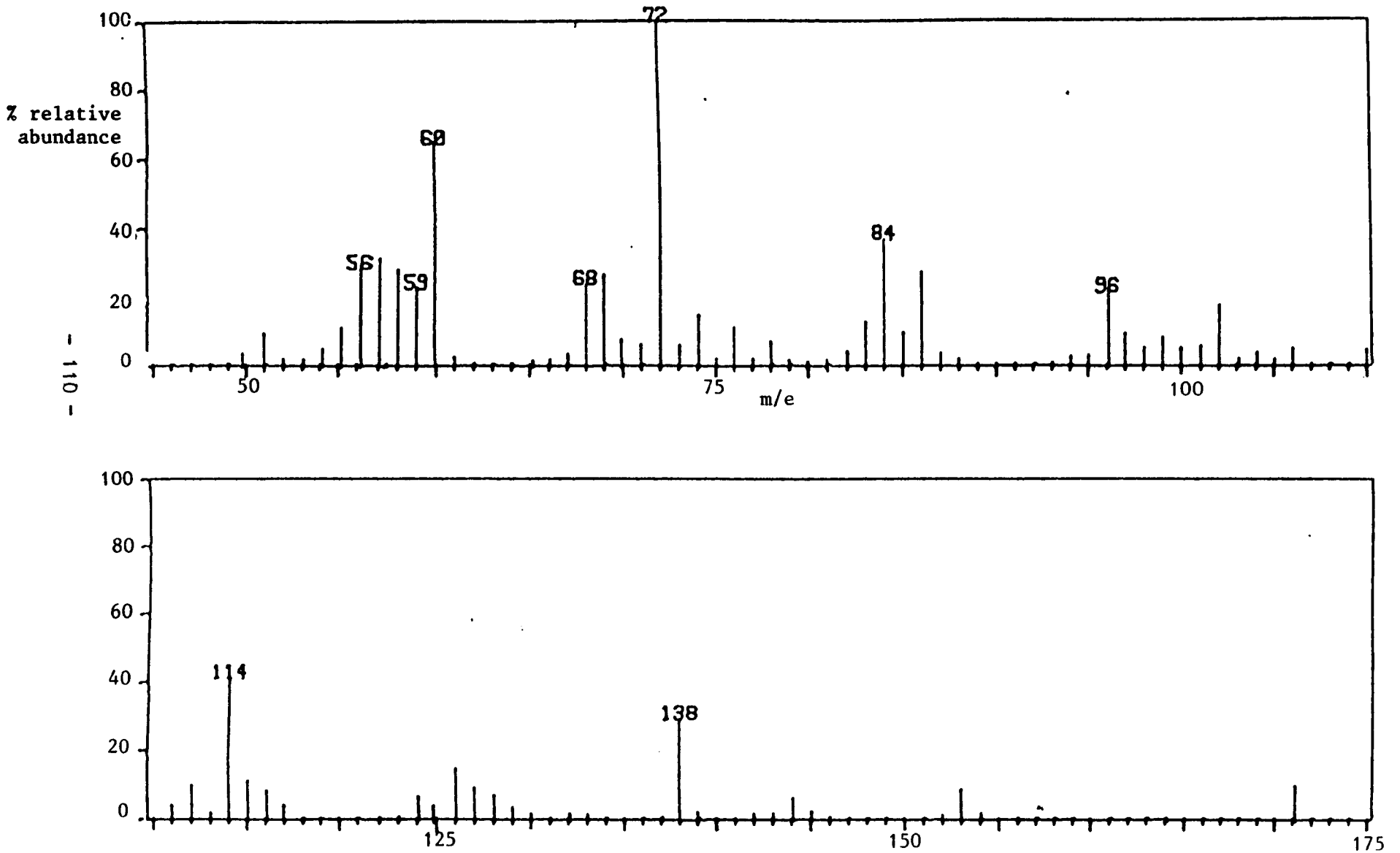


Figure 3.15: Mass spectrum of N-acetyldaunorubicin

3.2 Testing of daunorubicin and related compounds against *Trypanosoma rhodesiense in vitro* and *in vivo*

All testing for trypanocidal activity described in this section was performed by Dr. J. Williamson and Mr. T.J. Scott-Finnigan. (N.I.M.R., Mill Hill, London).

The *in vitro* screening test described in section 2.6.4 was developed for rapid screening of drugs for potential trypanocidal activity. In this simple assay method inhibition of motility has been shown to correlate well with inhibition of respiration and glycolysis (Williamson et al., 1975). Infectivity in mice has been used as an index of cell division as the monomorphic form of *Trypanosoma rhodesiense* used in this assay does not multiply *in vitro*. The two types of activity assessed indicate whether a trypanocidal agent is acting on energy producing reactions, or on macromolecular synthesis. A strong link between the anti-tumour and trypanocidal activity of drugs is well known (Kinnamon et al., 1979; Kandaswamy and Henderson, 1962), and this assay method was used to screen a number of anti-tumour agents for trypanocidal activity (Williamson and Scott-Finnigan, 1978, 1975) and revealed uniquely high activity for daunorubicin. The results of *in vitro* screening for a series of anthracycline antibiotics is presented in Table 3.3. High activity against trypanosomes, as shown by permanent loss of infectivity appears to be peculiar to daunorubicin, although the closely related drug doxorubicin, as well as nogalamycin and 7-O-methylnogarol show high temporary inhibition of infectivity. The locus of action appears to be macromolecular synthesis, rather than energy producing metabolism as only doxorubicin strongly inhibited motility. Strong inhibition of infectivity is expected as drug nuclear DNA association is believed to be involved in the cytotoxic action of daunorubicin (Henry, 1976).

Table 3.3: *In vitro* activity of anthracyclines and related compounds against *Trypanosoma rhodesiense*

Drug	Loss of motility [1]	Loss of infectivity [1]	
		Temporary	Permanent
Daunorubicin hydrochloride	4	11	10
5-Iminodaunorubicin hydrochloride	< 3	4	< 3
N,N-Dibenzyldaunorubicin hydrochloride	3	< 3	< 3
N-Acetyldaunorubicin #	< 3	5	< 3
4-Deoxydaunorubicin hydrochloride	5	6	3
4-Demethoxydaunorubicin hydrochloride	5	5	3
3,4-Diepidaunorubicin hydrochloride	5	> 10	> 10
Doxorubicin hydrochloride	8	> 10	4
Doxorubicin-14 stearate # hydrochloride	3	< 3	3
4-Deoxydoxorubicin hydrochloride	5	> 6	3
Nogalamycin	4	> 9	6
7-con-O-Methylnogarol	4	8	< 3
Daunorubicin-bovine serum * albumin (P)	4	4	< 4
Daunorubicin-bovine serum ** albumin (G)	< 4	> 10	> 10
Daunorubicin-ferritin (G) **	4	9	6

1 The values given are maximum titres ($\log_{10}M^{-1}$) producing the trypanocidal effect; drugs with titres < 3 are considered inactive.

* 'P' denotes that the periodate oxidation method (section 2.1.2) was used.

** 'G' denotes that the glutaraldehyde coupling method (sction 2.1.6) was used.

\# see section 2.2 for synthesis of these compounds.

In contrast to the wide range of activity shown by the anthracyclines *in vitro*, none of the drugs tested *in vivo* showed any activity (Table 3.4).

Table 3.4: Activity of anthracycline drugs in mice infected with *Trypanosoma rhodesiense*

Drug	Dose (mg/kg)	Number of mice used	Mean prolongation of infection (days)
Daunorubicin	30	2	0
Daunorubicin	20	2	0
Daunorubicin	15	15	0
Doxorubicin	30	4	0
5-Iminodaunorubicin	15	5	0.2
N-Acetyldaunorubicin	15	5	0
N-Acetyldaunorubicin	1.5	5	0

Brown et al. (1982) showed that uptake of daunorubicin is transitory, hence it was thought that increasing the retention of the drug by preparing a sustained release form of daunorubicin might lead to a trypanocidal effect. This was attempted by linking drug to a macromolecular carrier. Glutaraldehyde was chosen as a linking agent since Schiff's base linkages are known to be labile, and would be expected to readily release daunorubicin. Daunorubicin-bovine serum albumin conjugate linked via glutaraldehyde was active *in vitro* (Table 3.3) and also *in vivo* (Table 3.5). This conjugate prolonged the life of infected mice by several days, and also cleared trypanosomes from the bloodstream. However, no cures were obtained. In order to increase the efficacy of anthracycline conjugates three lines of research were explored which were: (a) variation of the macromolecular carrier; (b) variation of the drug-macromolecule linkage and (c) variation of the anthracycline used.

Table 3.5: Activity of glutaraldehyde linked daunorubicin macromolecule conjugates in mice infected with *Trypanosoma rhodesiense*

Conjugate	Dose of drug component (mg/kg)	Number of mice used (n)	Mean prolongation infection (days) ± (S.D.)
Bovine serum albumin	30	10	0.9 (0.5)[1,2]
" " "	15	10	2.9 (0.83)
" " "	7.5	10	3.9 (0.54)[1]
" " "	3.75	10	1.8 (0.6) [1]
" " "	1.88	10	1.2 (0.4)
Bovine serum albumin (Fraction 1)	15	7	3.85 (1.24)[1]
Bovine serum albumin (Fraction II)	15	5	5.0 (2.82)
Glutarated bovine serum albumin (2500 mg/kg)	None	7	0.0 (0)
Horse spleen ferritin	30	6	3.16 (2.11)[1,2]
" " "	15	10	4.1 (1.75)
" " "	7.5	1	4.0 (-)
" " "	3.8	1	4.0 (-)
" " "	1.9	1	1.0 (-)
Horse spleen apoferritin	15	1	3.0 (-)
Calf thymus histone f_1	12	1	3.0 (-)
Diaminopropyldextran	15	2	5.5 (0.5)

1 Trypanosomes cleared temporarily from the bloodstream.

2 Some deaths due to drug toxicity at this dose.

Table 3.6: Activity of daunorubicin-protein conjugates in mice infected with *Trypanosoma rhodesiense*

Daunorubicin Conjugate	Linkage	Dose of drug component (mg/kg)	Number of mice used	Mean prolongation of infection (days + S.D.)
Bovine serum albumin	Glutaraldehyde	15	10	2.9 (0.83)
Bovine serum albumin	Succinyl (amide)	15	4	0 (0)
Bovine serum albumin	Aminooxyacetic acid (oxime)	15	15	0.4 (0.8)
Bovine serum albumin	Hydrazinobenzoic acid (hydrazone)	15	4	0.5 (0.5)
Ferritin	Chelate	15	10	0.2 (0.4)
Dextran	Periodate oxidation	15	1	0 (0)

These conjugates were weakly active, prolonged the infection of only a few of the mice tested, and did not clear trypanosomes from the bloodstream of the infected mice.

Glutaraldehyde-linked bovine serum albumin conjugate, prepared using anthracyclines other than daunorubicin, were also active (Table 3.7), although none was as active as daunorubicin.

Table 3.7: Activity of anthracycline-bovine serum albumin conjugates in mice infected with *Trypanosoma rhodesiense*.

Drug	Dose of drug component (mg/kg)	Number of mice used	Prolongation of infection (days ± S.D.)
Daunorubicin	15	10	2.9 (.83)
4-Demethoxydaunorubicin	15	10	2.4 (.916)
4-Deoxydaunorubicin	15	3	2.0 (1.4)
5-Iminodaunorubicin	15	5	2.0 —
	7.5	5	0.0 (2)
Doxorubicin	15	10	1.5 (0.5)
4-Deoxydoxorubicin	15	2	0.5 (0.5)
3,4-Diepidaunorubicin	15	1	1.0 (0)

Daunorubicin was the most potent anthracycline drug tested *in vitro*, but all of the anthracyclines were inactive *in vivo*. However, as a glutaraldehyde-linked conjugate, daunorubicin was also the most active drug tested, but there was no obvious relationship between *in vitro*, activity of the drugs and the *in vivo* activity of the glutaraldehyde linked conjugates. Glutaraldehyde-linked daunorubicin bovine serum albumin conjugate cleared trypanosomes from the bloodstream of infected mice and prolonged the course of infection, but no cures

were obtained. Linking agents, other than glutaraldehyde, produced conjugates which were only weakly active *in vivo*.

The strain of *T. rhodesiense* used in these experiments has been maintained by syringe passage for 35 years (Yorke et al., 1929) and consists solely of long, thin, forms. This strain cannot be considered representative of wild strains, hence daunorubicin-BSA-conjugate was also tested against a variety of trypanosome strains. The results are given in Table 3.8

Table 3.8: Activity of glutaraldehyde-linked daunorubicin bovine serum albumin in mice infected with various trypanosome strains.

Trypanosome strain	Dose (mg/kg)	Number of mice used	Mean prolongation of infection (days)
T. brucei (427)	15	10	6.4
"	7.5	8	5.6
" (serengeti)	3.7	10	3.0
" (serengeti)	15	10	0.3
T. rhodesiense (Stilbamidine resistant)	15	10	2.8
"	7.5	10	9.8
T. congolense	15	10	3.8

Glutaraldehyde-linked daunorubicin-BSA conjugate was active against all strains of trypanosome tested. The strain used did have an effect on the prolongation of infection, since the conjugate was only slightly active against *T. brucei* (serengeti) strain. Infection was prolonged to at least 18 days longer than untreated controls, but no cures were obtained. Cures would be expected, since this conjugate clears trypanosomes from the bloodstream. Abolarin et al. (1982) showed that

T. brucei enter the ependymal cells of the choroid plexus, which constitutes part of the blood-brain-barrier. This might protect the intracellular trypanosomes from the effect of macromolecule conjugates since neither macromolecules nor freedaunorubicin will cross the blood-brain-barrier (May et al., 1980). The intracellular form may re-enter the bloodstream and re-establish the infection. This intracellular form may restrict the usefulness of conjugates in the treatment of this disease. Similar intracellular forms are present in Chagas disease and have prevented the development of effective chemotherapeutic agents.

3.3 *In vitro* breakdown of daunorubicin-macromolecule conjugates in biological fluids

Daunorubicin-macromolecule conjugates have been evaluated in numerous cancer studies (Levi-Schaffer et al., 1982; Hurwitz et al., 1975; Bernstein et al., 1978; Monsigny et al., 1980). However, little attention has been paid to the stability of the linkage between drug and carrier. *In vitro* and *in vivo* testing of a variety of daunorubicin-macromolecule conjugates (section 3.2) revealed that conjugates differing only in the type of linkage used may have widely differing activities against *T. rhodesiense*. The nature of the linkage is thus of great importance in understanding the way in which conjugates exert their cytotoxic effects. Daunorubicin macromolecule conjugates (linked via glutaraldehyde) may be postulated to act (i) to transport daunorubicin prior to release of free drug in the bloodstream or after ingestion by the parasite, and/or (ii) by acting as a trypanocide without release of drug. There is, therefore, a need to investigate whether drug can be released under physiological conditions in order to more fully understand the mechanism of action and, hence, design more effective conjugates.

Methods for the determination of daunorubicin and its metabolites have usually involved extraction from a basified sample. Determination has been by HPLC using fluorescence detection (Hulhoven and Desager, 1976; Israel et al., 1978; Brown et al., 1981). A method was developed based on extraction from a basified solution followed by fluorescence detection (see section 2.3.4). Calibration curves were prepared by extraction of known concentrations of daunorubicin from phosphate buffered saline and murine plasma. The extraction efficiency was taken as 100% since following the extraction procedure the remaining solution of phosphate buffered saline did not absorb light at the λ_{max} of daunorubicin (495 nm). Extraction of daunorubicin from murine plasma or trypanosome homogenate could not be tested in this way due to light scattering by the solution. Linear calibration was obtained and regression analysis gave correlation coefficients greater than 0.997 (see Figure 3.16).

Daunorubicin release from conjugates in phosphate buffered saline, pH 7.4, is illustrated in Figure 3.18.

The fraction of drug released from daunorubicin-bovine serum albumin conjugate linked via glutaraldehyde depended on the concentration (μg/mg protein) of drug initially linked. For example, the conjugate containing 13.3 μg/mg protein released 12% of bound drug after two and a half hours, but conjugate containing 3.9 μg/mg protein only released 5% of bound drug in the same time period (see Figure 3.18). In either case, no further drug was released, even after a five-hour incubation period.

Glutaraldehyde conjugate was isolated after prolonged incubation as described in section 2.1.6.c, and free drug was removed by passage through a Porapak Q column. This conjugate, from which weakly bound

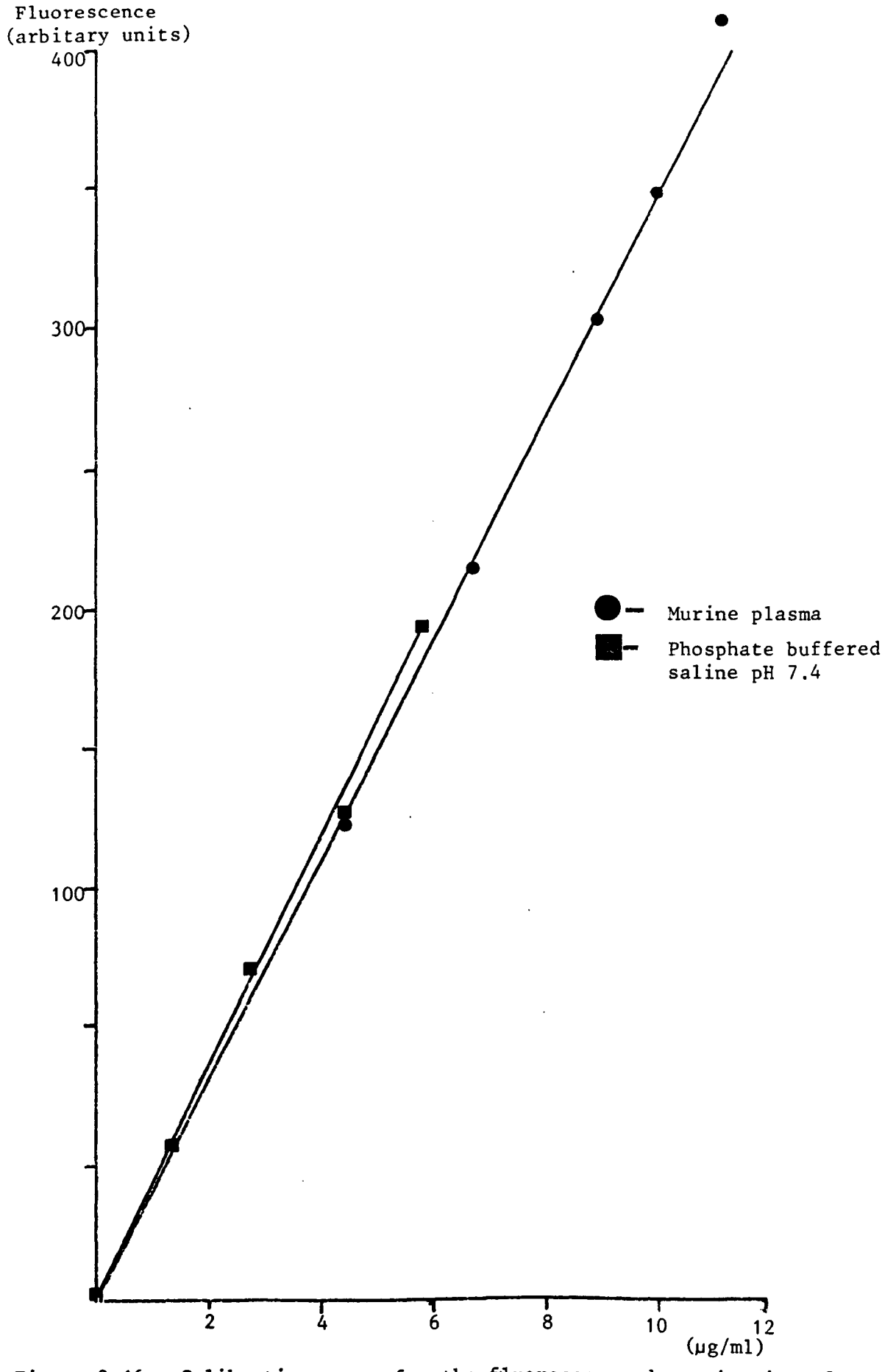


Figure 3.16: Calibration curve for the fluorescence determination of daunorubicin in PBS and murine plasma.

drug had been removed, was called fraction II glutaraldehyde conjugate, the material prior to incubation being called fraction I conjugate.

As expected, there was no release of drug from fraction II glutaraldehyde conjugate when further incubated in phosphate buffered saline (Figure 3.18). Drug release from conjugates in murine plasma is illustrated in Figure 3.19. Fraction I glutaraldehyde conjugate released about 21% of bound drug, while fraction II conjugate released about 9%. This fresh drug release of fraction II conjugate is presumably due to enzymic hydrolysis of the daunorubicin-glutaraldehyde bond. Incubation of conjugates in trypanosomal homogenates gave similar results to those found for murine plasma (Figures 3.19 and 3.20). However, fraction I conjugate released about 35% of bound drug, while fraction II glutaraldehyde conjugate released about 10% of bound drug. These results demonstrate that the glutaraldehyde conjugate of daunorubicin-BSA must contain at least two different bond types, since drug which is not released in phosphate buffered saline is released in murine plasma and trypanosomal homogenate. In support of this, Richards and Knowles (1968) showed that glutaraldehyde exists as an α, β-unsaturated aldehyde in aqueous solution and reacts with amino groups to give a stable Michael-type adduct, as well as linkages containing Schiff's base bonds (Figure 3.17).

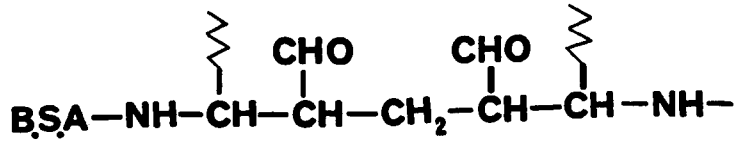


Figure 3.17: Proposed structure of the glutaraldehyde bond (Richards and Knowles, 1968).

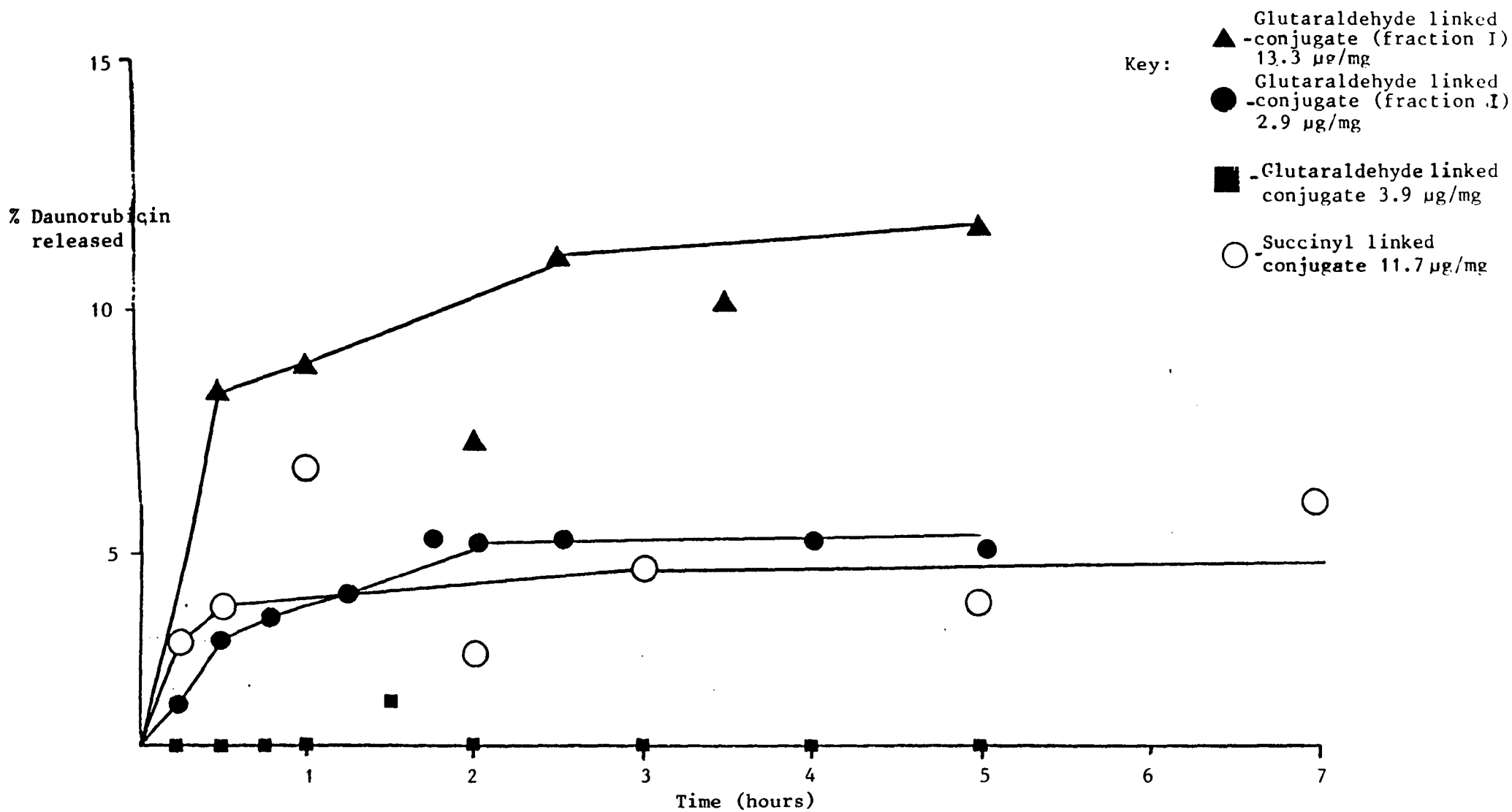


Figure 3.18: Release of daunorubicin from bovine albumin conjugate when incubated in PBS pH 7.4 at 37°C

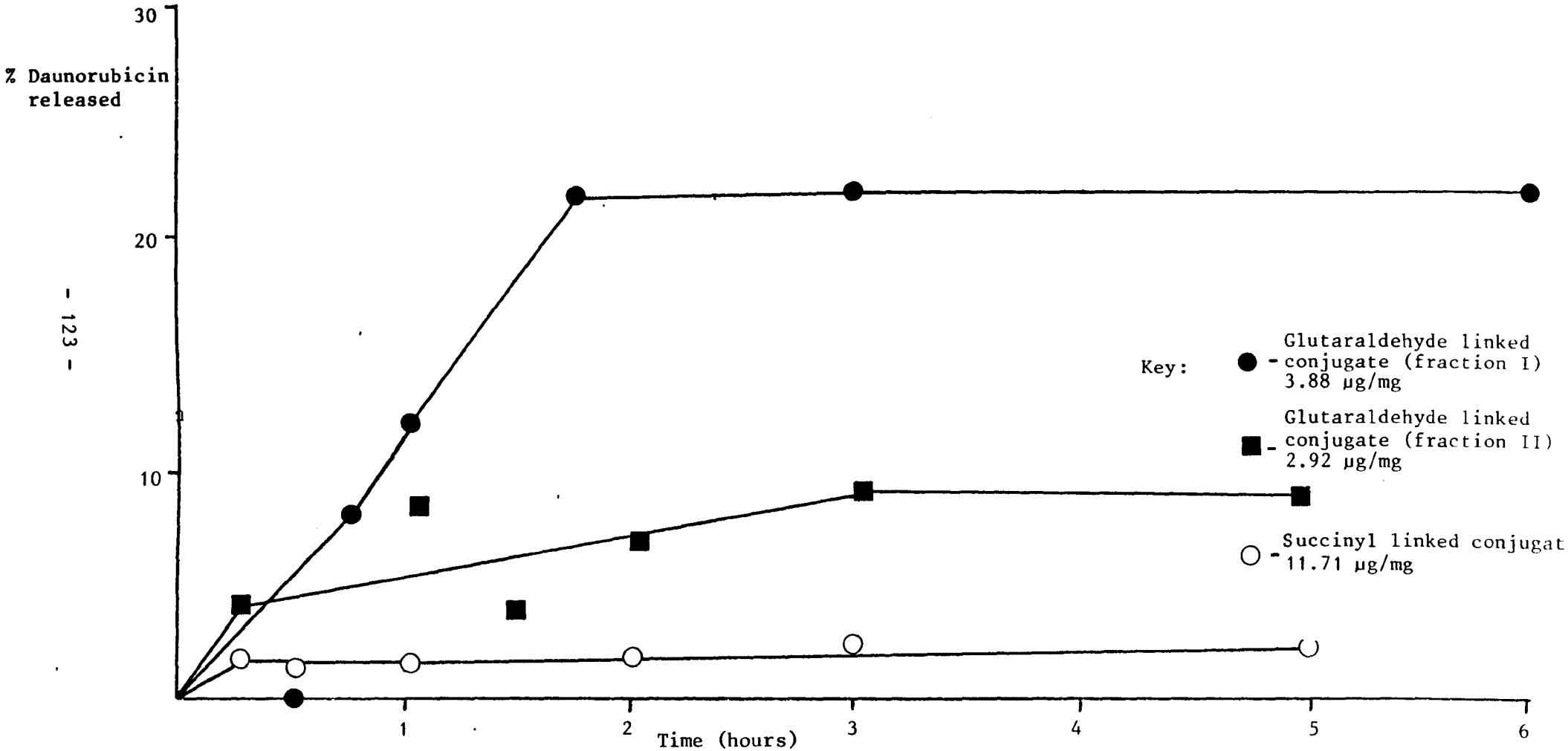


Figure 3.19: Release of drug from daunorubicin-bovine serum albumin conjugates when incubated in murine plasma at 37°C

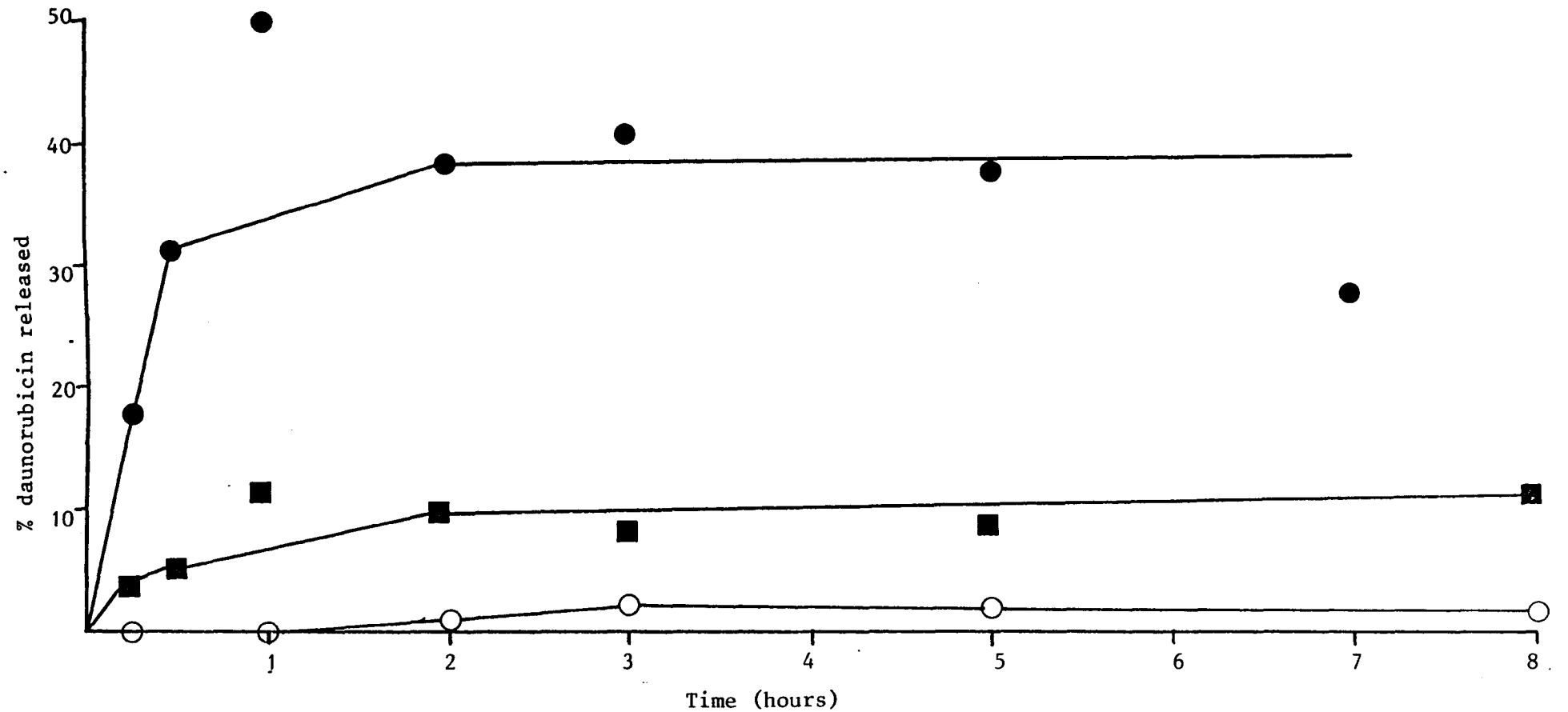


Figure 3.20: Release of drug from daunorubicin-bovine albumin conjugates incubated in trypanosomal homogenate at 37°C

Even after incubation in murine plasma and trypanosomal homogenate (Figures 3.19 and 3.20), not more than 35% of bound drug is released. The remaining drug may be linked to protein by a bond which is not susceptible to hydrolysis or is sterically hindered towards enzyme mediated hydrolysis.

Fraction I and fraction II conjugate were both active against *Trypanosoma rhodesiense in vivo*. Fraction II was more active than fraction I, which suggests that the enzymically released component is important in the activity of these conjugates.

Succinyl linked daunorubicin-bovine serum albumin was more stable to drug release than the fraction I glutaraldehyde conjugates and released about 3% of the total bound drug in phosphate buffered saline, murine plasma and trypanosomal homogenate. This result is in close agreement with that of Trouet et al. (1982) who showed that a daunorubicin-succinyl-bovine serum albumin conjugate containing 100 µg drug/mg protein released a total of 2.6% of bound drug when incubated in the presence of purified rat liver lysosomes. The high stability to drug release of this conjugate is expected since the amide bond is probably protected from attack by hydrolytic enzymes due to steric hindrance by the carrier protein. In addition, the amide bond is not in an α position with regard to an asymmetric carbon atom, which makes it a poor substitute for peptidases and proteases (Trouet et al., 1982b). In the present study, it would appear that drug release from the succinyl-linked daunorubicin-BSA is not enzymically mediated since the same proportion of bound drug is released in all three media tested.

The breakdown of the hydrazone linked conjugate is illustrated in Figure 3.21. The linkage was highly labile, releasing most of

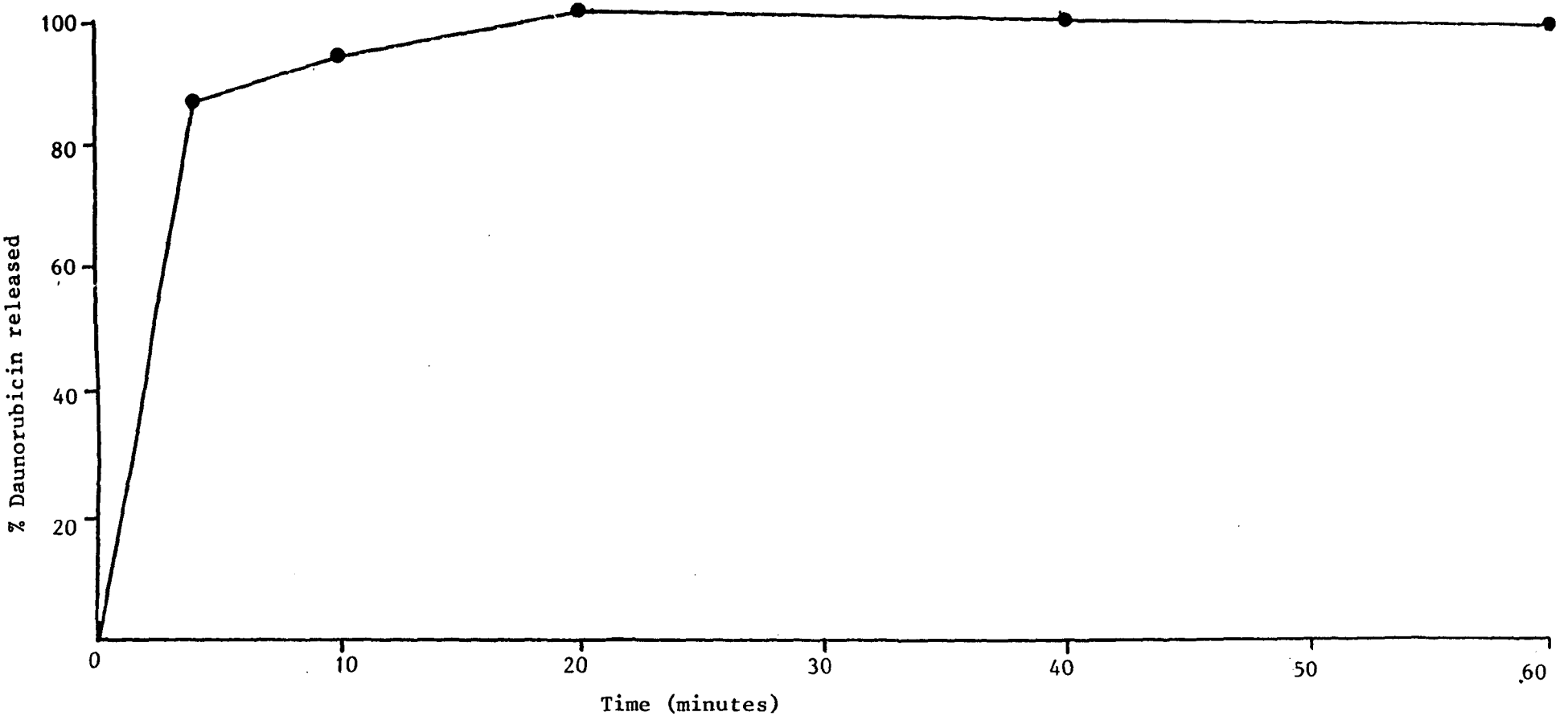


Figure 3.21: Release of hydrazone-linked daunorubicin-bovine serum albumin conjugates (7.2 μg mg^{-1}) incubated in phosphate buffered saline at 1°C (approx.)

the bound drug within a few minutes. Oxime linkage also released drug very rapidly, but the rate of breakdown could not be accurately estimated. The highly labile oxime and hydrazone linked conjugates and the refractory succinyl conjugate were all inactive (see section 3.2). Hence, it appears that an intermediate rate of release is required for activity and that this requirement is fulfilled by glutaraldehyde linkage. Drug release from glutaraldehyde conjugate is complete in two to three hours in murine plasma and trypanosomal homogenate, whereas hydrazone and oxime linked conjugates released 100% of bound drug within one hour. Succinyl linked conjugate released about 2% of drug within fifteen minutes, but little was released thereafter.

Glutaraldehyde conjugate may be acting as a sustained release form of daunorubicin, since a trypanocidal dose of 25 mg/kg will release at least 12%, or 3 mg/kg of free drug over a three-hour period. This is many times greater than the trypanocidal concentration of 0.05 mg/L required for *in vitro* activity (Williamson and Scott-Finnigan, 1978). However, Brown et al. (1982) demonstrated that a daunorubicin dose of 25 mg/kg daunorubicin *in vivo* produces a drug concentration in blood plasma of 500 times the *in vitro* trypanocidal concentration over a six-hour period, although this dose is not trypanocidal *in vivo*. It is, therefore, unlikely that the *in vivo* activity of glutaraldehyde conjugates is solely due to sustained release of drug in the plasma.

In addition to considering release of drug from the conjugate, it is important to consider whether the conjugate may be acting as a drug without prior release of daunorubicin. This hypothesis has received support in recent years. Tokes et al. (1982) found that cytotoxic doxorubicin-polyglutaraldehyde microspheres could interact with DNA, and Tritton and Yee (1982) found that doxorubicin linked to microspheres was cytotoxic, although no drug was released.

3.4 Interaction of daunorubicin analogues with DNA

The anthracycline drugs are known to bind avidly to DNA by intercalation into the DNA double helix (Brown, 1978), and to inhibit DNA synthesis in cell-free systems (Zunino et al., 1975) as well as *in vivo* (Di Marco et al., 1972). Hence chromosomal DNA has been assumed to be their site of action. DNA binding is accompanied by changes in the physical properties of both drug and DNA, and these changes can be used to assess the strength of the binding. On intercalation, there is a real shift in the visible absorption spectrum of the drug (bathochromic shift), a decrease in the extinction coefficient hypochromic shift), and a decreased bathochromic shift on basification, due to the drug passing from a hydrophilic to a hydrophobic environment (Lawrence, 1952). The fluorescence of the drug is also markedly quenched after binding to DNA (Lober and Kittler, 1978). Changes in the physical properties of DNA which occur on intercalation include an increase in thermal denaturation temperature due to stabilisation of the DNA double helix by intercalated drug molecules (Kersten et al., 1966), and increased viscosity (Zunino et al., 1972). Intercalation also causes unwinding of circular supercoiled DNA (Waring, 1970).

The effect of DNA on the spectral properties of a series of daunorubicin analogues is shown in Table 3.9. The spectral changes which occurred can be divided into two groups, depending on whether the changes are similar to, or different from, daunorubicin, which is known to intercalate. Drugs which had similar DNA spectral properties to daunorubicin include 5-iminodaunorubicin and nogalamycin. All of these drugs showed an isosbestic point, large bathochromic (9-14 nm) and hypochromic (21-32%) shifts. They also showed a large shift on basification in the absence of DNA, but a small shift in the presence

Table 3.9: Effect of DNA on the spectral properties of daunorubicin analogues

Drug	Position of isosbestic point (nm) (1)	Bathochromic shift (nm) (1)	% Decrease in extinction coefficient (1)	Bathochromic shift on basification (nm) (1),(3)	
				DNA present	DNA absent
Daunorubicin	542	12	32	0	29
5-Iminodaunorubicin	597	14	21	3	10
N-Acetyldaunorubicin	540	3	7.5	19	18
N,N-Dibenzyldaunorubicin (2)	-	10	-7 (4)	1	1
Daunorubicin-BSA (fraction II) (2)	-	0	4	7	4
Nogalamycin	512	9	24	11	40
7-O-Methylnogarol	-	3	6	22	27

(1) Drug concentration 5×10^{-5}M, DNA/drug ratio 10:1

(2) Drug concentration 2.5×10^{-5}M, DNA/drug ratio 10:1

(3) pH of 7.4 and 9.4 used

(4) 7% enhancement of absorbance

Figure 3.22: Spectral titration of daunorubicin-BSA conjugate with calf thymus DNA. DNA/drug ratios of 0, 1:1, 2:1, 5:1, 10:1. Daunorubicin concentration 2.5×10^{-5}M.

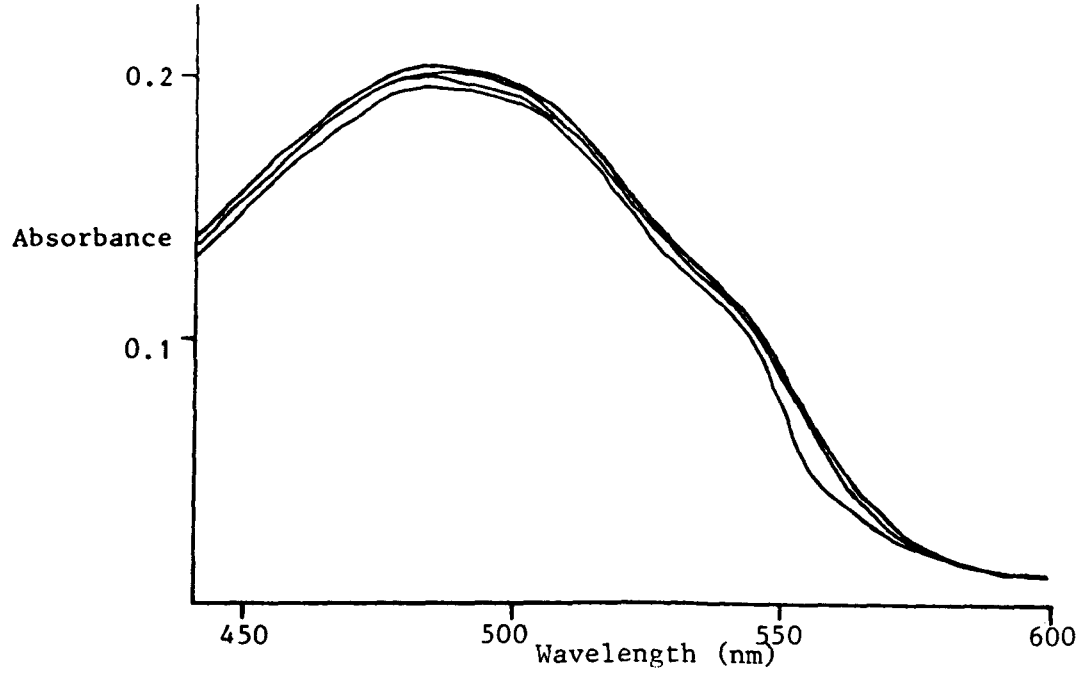
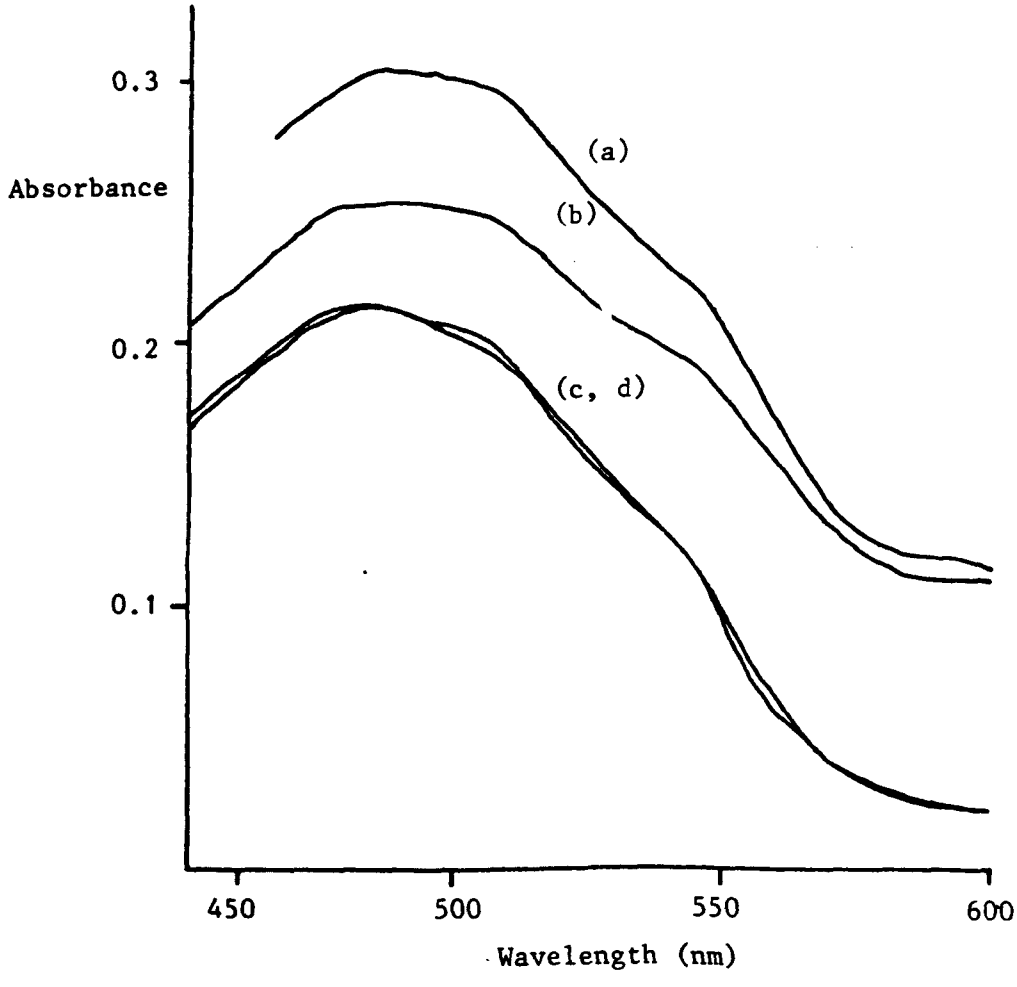


Figure 3.23: pH shift of daunorubicin-BSA conjugate on basification in the presence and absence of calf thymus DNA (a) pH 9.4 no DNA, (b) pH 9.4 DNA present, (c) pH 7.4 DNA present, (d) pH 7.4 no DNA present.



of DNA. Drugs which reacted differently from daunorubicin showed little change in spectral properties in the presence of DNA. This group of drugs included N,N-dibenzyldaunorubicin, 7.-O-methylnogarol, N-acetyldaunorubicin and daunorubicin BSA conjugate (fraction II). With the exception of N-acetyldaunorubicin, these drugs did not show an isosbestic point, but showed small bathochromic (0-10 nm) and hypochromic shifts (0-7.5%). Bathochromic shifts on basification were little changed in the presence of DNA. In contrast to daunorubicin, the spectral interaction of daunorubicin-BSA conjugate (fraction II) shows no isosbestic point, or significant hypochromic or bathochromic shift (Figure 3.24) and the bathchochromic shift on basification is greatest in the presence of DNA. Daunorubicin does not show any bathochromic shift under these conditions. On the basis of these tests, there is no evidence that daunorubicin-BSA conjugate interacts with DNA. This conclusion is supported by the results of Weisenhahn et al. (1977) who used spectral tests to demonstrate that a daunorubicin-melanotropin conjugate did not interact with DNA.

The interaction between drug and DNA can be investigated by fluorescence titration since quenching of fluorescence in the presence of DNA is known to be indicative of a DNA-drug interaction (Calendi, et al. 1965).

The results of fluorescence titrations are shown in Figures 3.26 and 3.27. Drugs which show marked quenching in the presence of DNA include daunorubicin, 5-iminodaunorubicin, nogalamycin and 7-O-methylnogarol. N-Acetyldaunorubicin and N,N-dibenzyldaunorubicin did not show this quenching of fluorescence on addition of DNA. The quenching observed with 7-O-methylnogarol is an anomaly, since, in all other tests used, this drug behaved differently to daunorubicin.

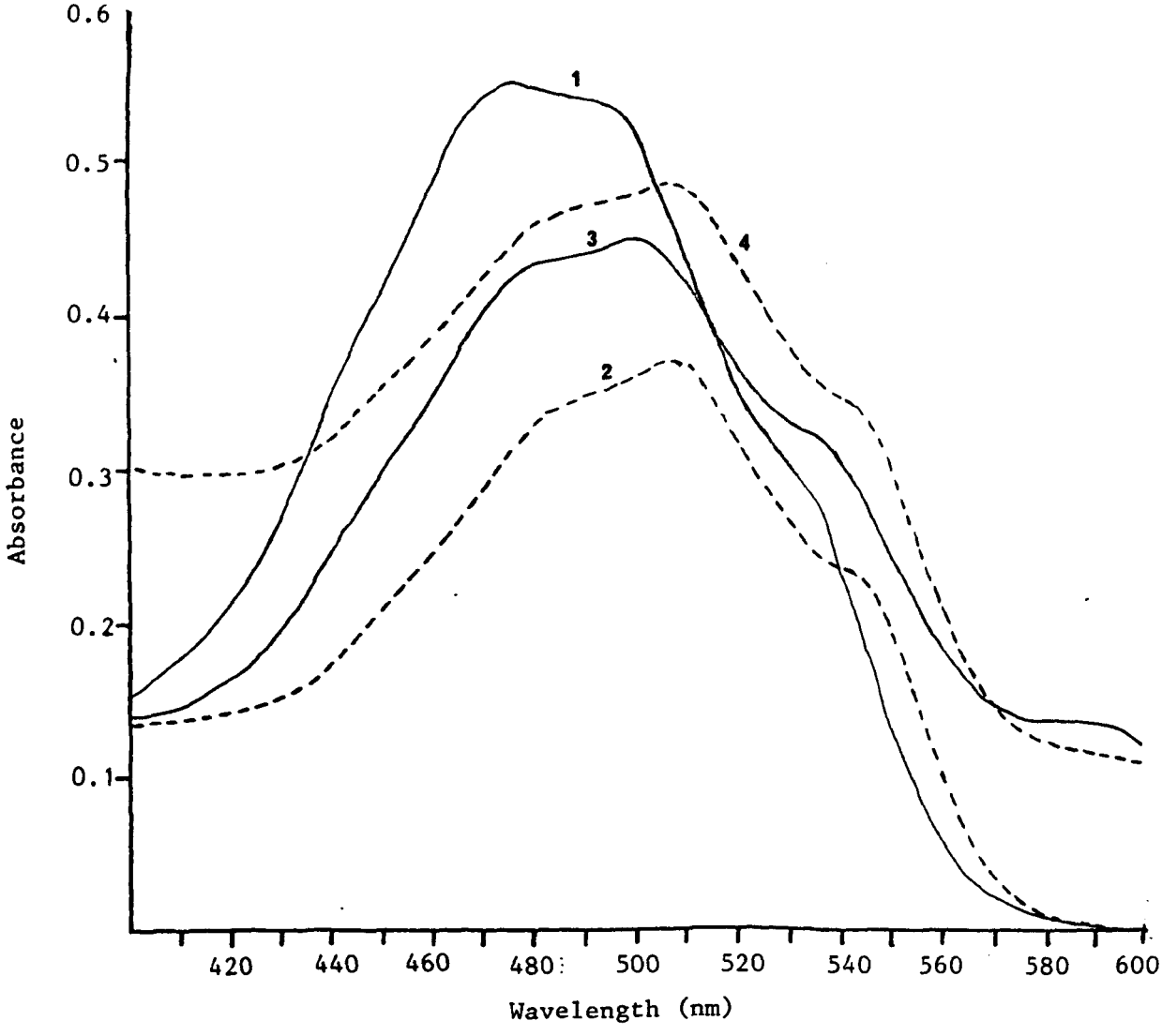


Figure 3.24: pH shift of daunorubicin. Drug concentration 5 x 10^{-5}M DNA/drug ratio 10:1.

(1) pH 7.4, (2) pH 7.4 DNA present, (3) pH 9.4

(4) pH 9.4 DNA present.

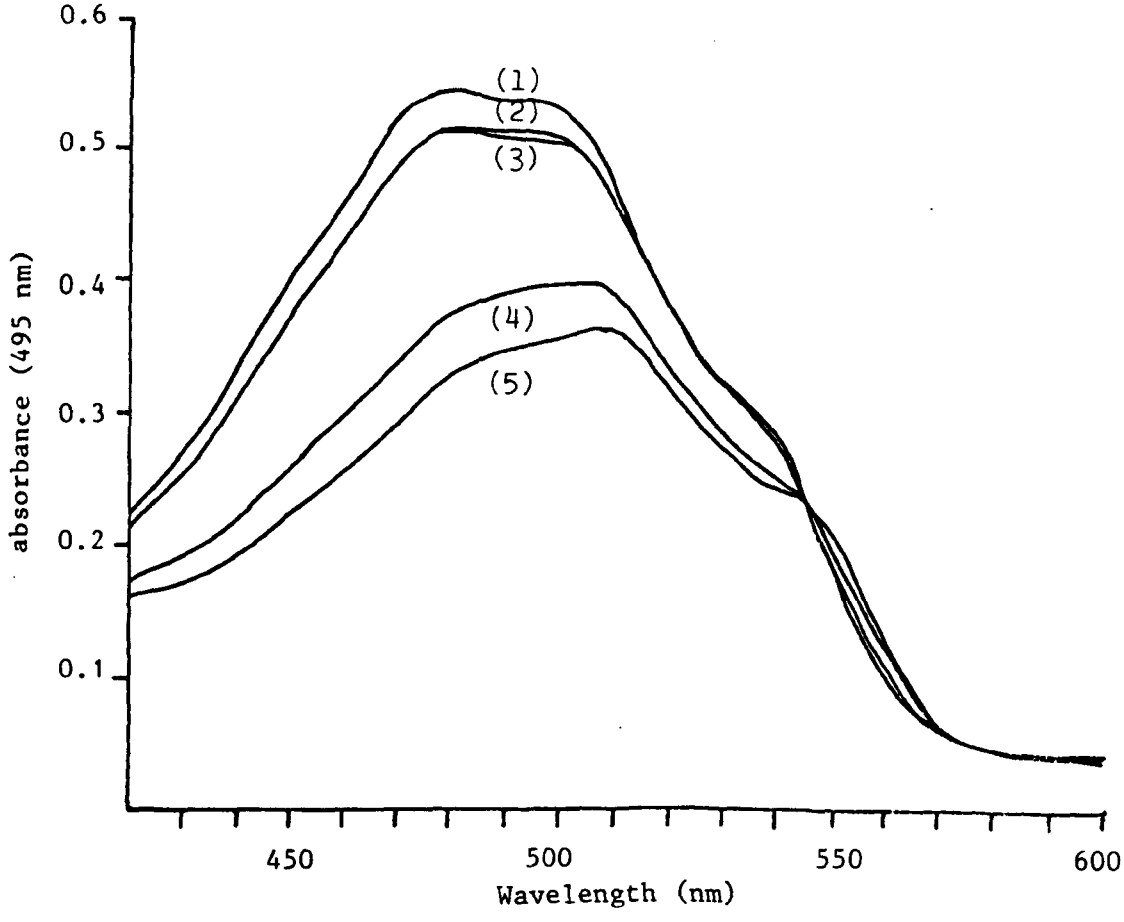


Figure 3.25: Spectral titration of daunorubicin with calf thymus DNA. Drug concentration 5 x 10^{-5}M. DNA/drug ratio (1) 0:1, (2) 1:1, (3) 2:1, (4) 5:1, (5) 10:1.

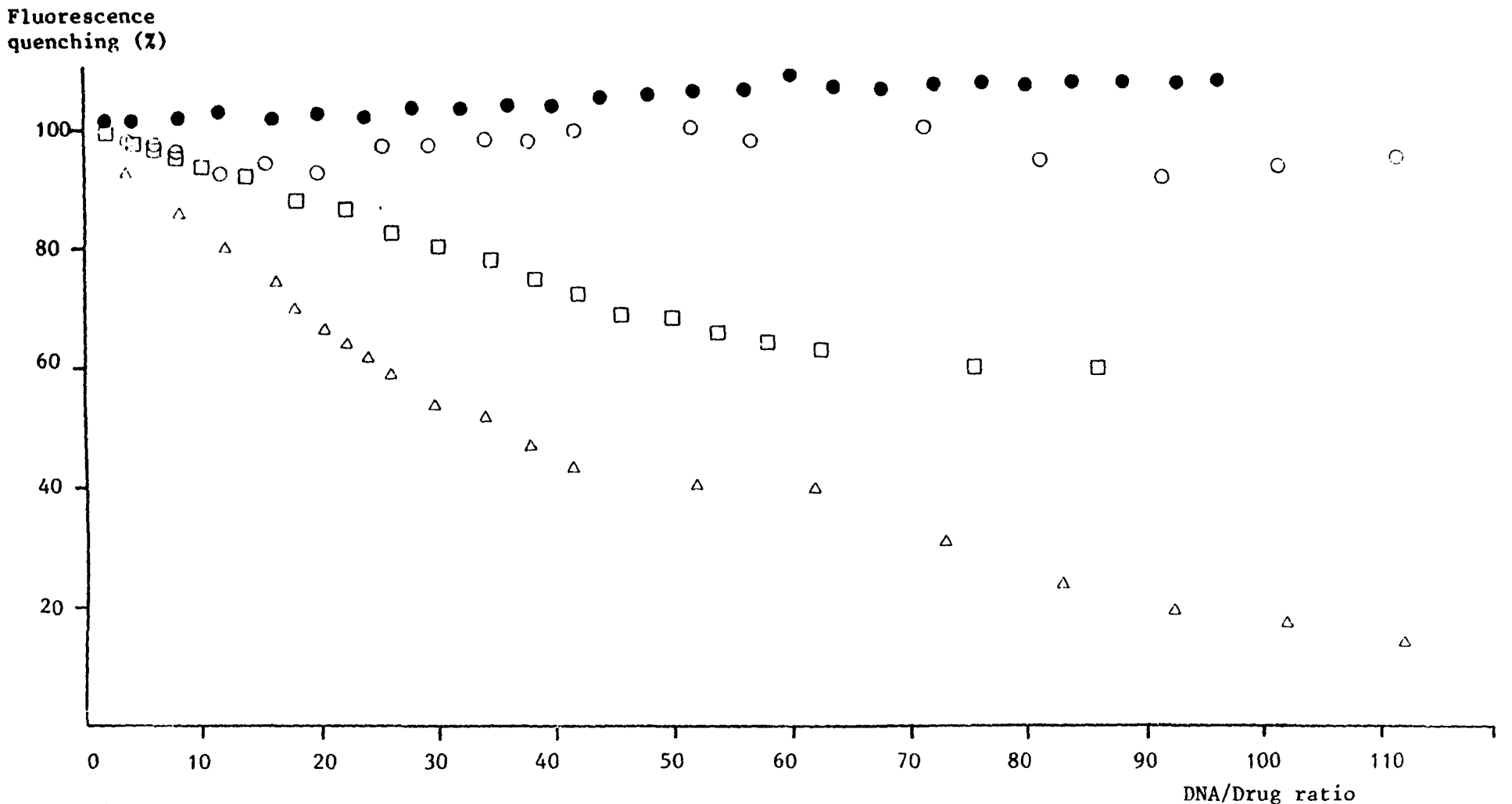


Figure 3.26: Fluorescence titration of calf thymus DNA with anthracycline drugs
○- N,N-dibenzyldaunorubicin, □- 5-iminodaunorubicin, △- daunorubicin,
●- N-acetyldaunorubicin

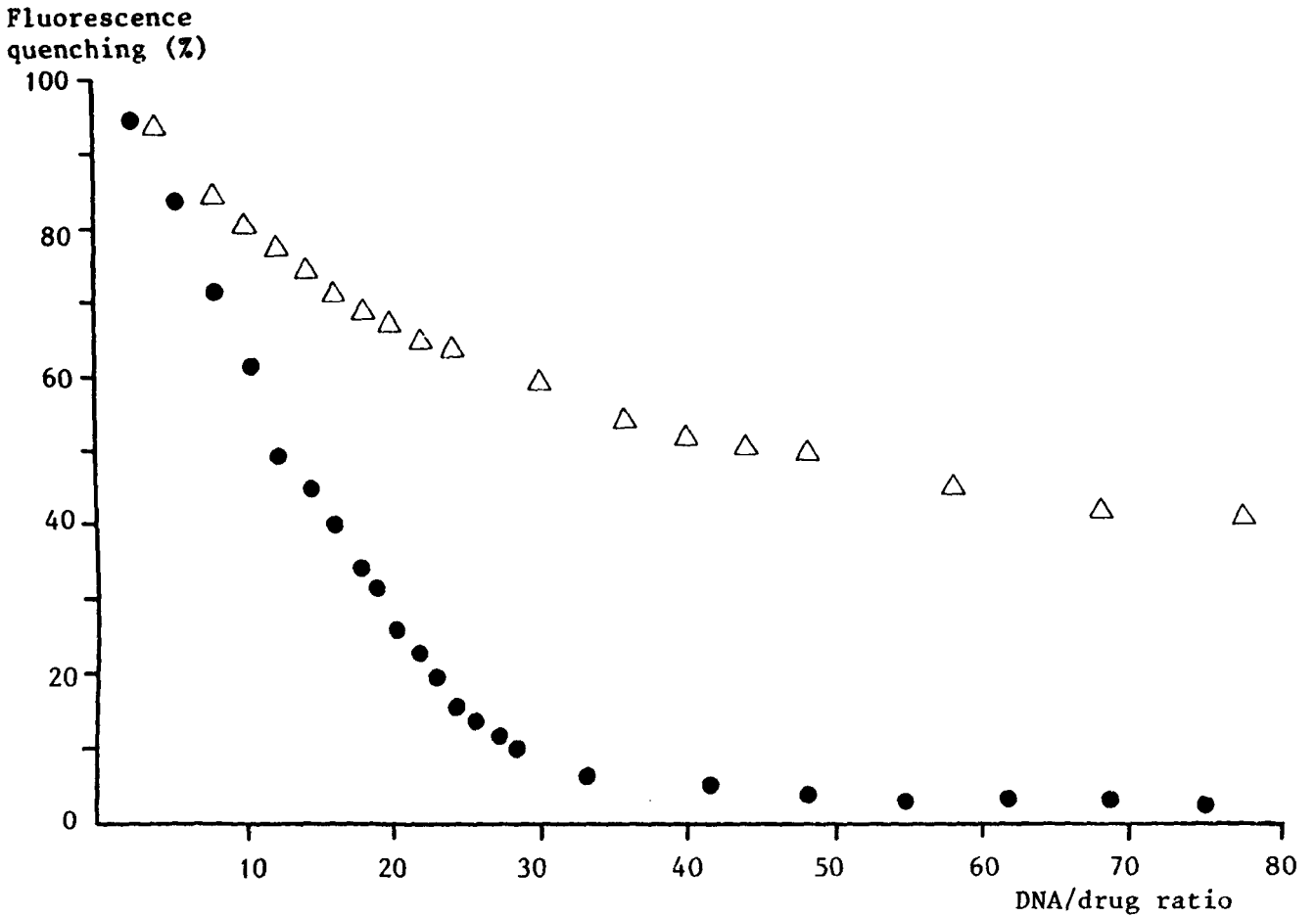


Figure 3.27: Fluorescence quenching of nogalamycin and 7-0 methylnogarol in the presence of calf thymus DNA. △ - 7-0 methylnogarol, ● - nogalamycin

A similar anomaly has been observed with the 7-R analogue of daunorubicin which does not intercalate, but undergoes fluorescence quenching in the presence of DNA (Plumbridge and Brown, 1979).

With the exception of bathochromic shifts on basification in the presence and absence of DNA, spectral tests do not unequivocally reveal the nature of the DNA drug interaction, since other modes of binding, such as electrostatic binding to the exterior of the double helix (Neidle, 1981) might also produce these phenomena. There is therefore a need for confirmatory tests to indicate whether the binding observed is intercalation.

Confirmatory tests used in this study include thermal stabilisation of DNA in the presence of drug and the effect of drug on the electrophoretic mobility of supercalated circular DNA. These tests measure changes in the physical properties of DNA and are independent of the spectral properties of the drug. These changes are, with very few exceptions (Waring, 1970), specific for an intercalation mode of interaction.

The interaction of daunorubicin with DNA stabilises the double helical structure of the macromolecule to thermal denaturation (Zunino et al., 1972). The results of the thermal denaturation study are presented in Table 10. Since the extinction coefficient of denatured DNA at 260 nm is greater than that of native DNA, the rate of thermal denaturation can be followed spectrophotometrically as the temperature of the sample is raised. This produces a sigmoid curve (Figure 3.28); the thermal denaturation temperature Tm is taken as the mid-point of the curve. The values of ΔTm obtained in this study are closely similar to values previously obtained by other workers (Table 3.10). The thermal denaturation temperature

Table 3.10: Increase in thermal denaturation temperature (Δ Tm) of calf thymus DNA in the presence of anthracycline drugs. (0.003M tris, 0.018M NCl pH 7.4 buffer used, DNA/drug ratio 10:1)

Drug	Tm (°C)	Literature value (°C)	Reference
Daunorubicin	14.8 ± 1.2	13.4 (1)	Zunino et al. (1972)
5-Iminodaunorubicin	9.1 ± 0.1	6.5 (2)	Plumbridge & Brown (1978)
N-Acetyldaunorubicin	2.1 ± 0.5	1.0	Zunino et al. (1979)
N,N-Dibenzyldaunorubicin	0.6 ± 0.0	1.35	Tong et al. (1979(b)
Nogalamycin	14.2 ± 0.5	20 (3)	Das et al. (1974)
		17.5	Plumbridge & Brown (1979)
7-O Methylnogarol	1.75 ± 0.05	-	-

(1) 10:1 DNA/drug ratio in 0.01M Tris pH 7.0 buffer

(2) 10:1 DNA/drug ratio in 0.003M Tris, 0.018 NaCl pH 7.0 buffer

(3) 12:1 DNA/drug ratio in 0.02M ionic strength buffer

(4) 10:1 DNA/drug ratio in 0.01M phosphate, 0.001M EDTA pH 6.0 buffer

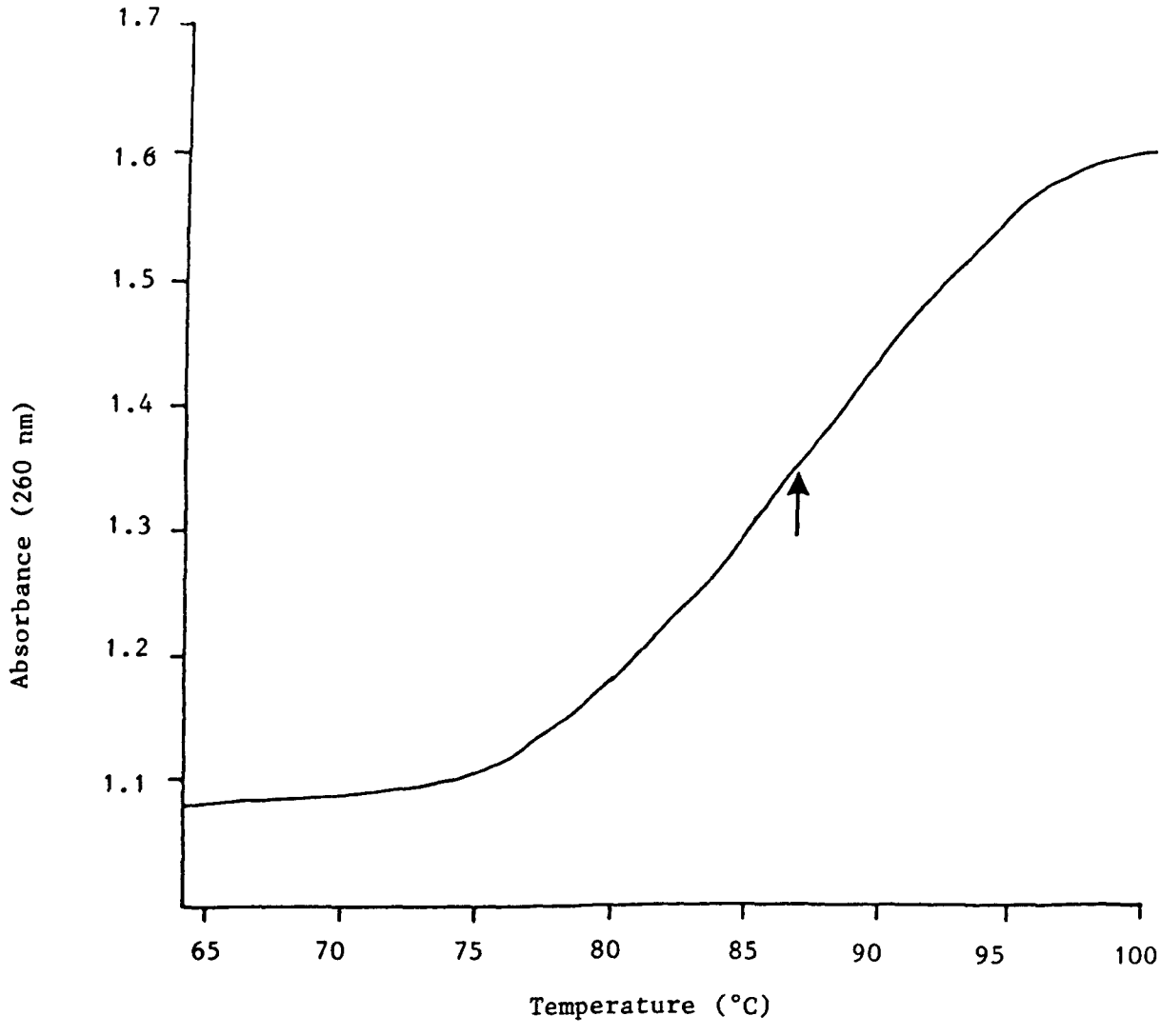


Figure 3.28: Thermal denaturation of DNA in the presence of daunorubicin (Thermal denaturation temperature Tm, is taken as the temperature corresponding to the mid-point of the absorbance/temperature sigmoidal curve.)

is affected by the DNA/drug ratio and the ionic strength of the buffer used, and these factors must be considered when comparing the results with earlier work. Significant thermal denaturation of DNA occurs in the presence of daunorubicin and other drugs which are thought to intercalate. N-acetyldaunorubicin, N,N-dibenzyldaunorubicin and 7-O methylnogarol which in spectral tests showed little evidence of interaction with DNA, also showed little effect on the thermal stability.

The local unwinding of the DNA double helix which occurs on intercalation has important consequences when the DNA is circular. Covalently closed circular DNA is supercoiled, which makes the molecule unusually compact so that it has a high sedimentation coefficient and electrophoretic mobility, but a low viscosity (Neidle, 1981). When intercalation occurs, unwinding of the DNA relaxes the supercoiling until at a critical drug concentration the DNA molecule behaves as the relaxed, open circular form. This change is accompanied by large changes in viscosity, sedimentation coefficient and electrophoretic mobility (Waring and Henley, 1975; Espejo and Liebowitz, 1976). As further drug binding occurs, the additional unwinding forces the DNA molecule to adopt reverse supercoils, so that the sedimentation coefficient and electrophoretic mobility rise, and the viscosity falls. This behaviour is indicative of intercalation since drugs such as cyclophosphamide and berenil, which bind to DNA but do not intercalate, do not show these changes (Waring, 1970; Mong et al., 1979). An electrophoretic titration of covalently closed circular PM2-DNA in the presence of an intercalating drug produces a result similar to Figure 3.29. At the critical drug concentration, the upper band of closed circular DNA co-migrates with relaxed nicked circular DNA (lower band). In contrast, Figure 3.30 shows an electrophoretic

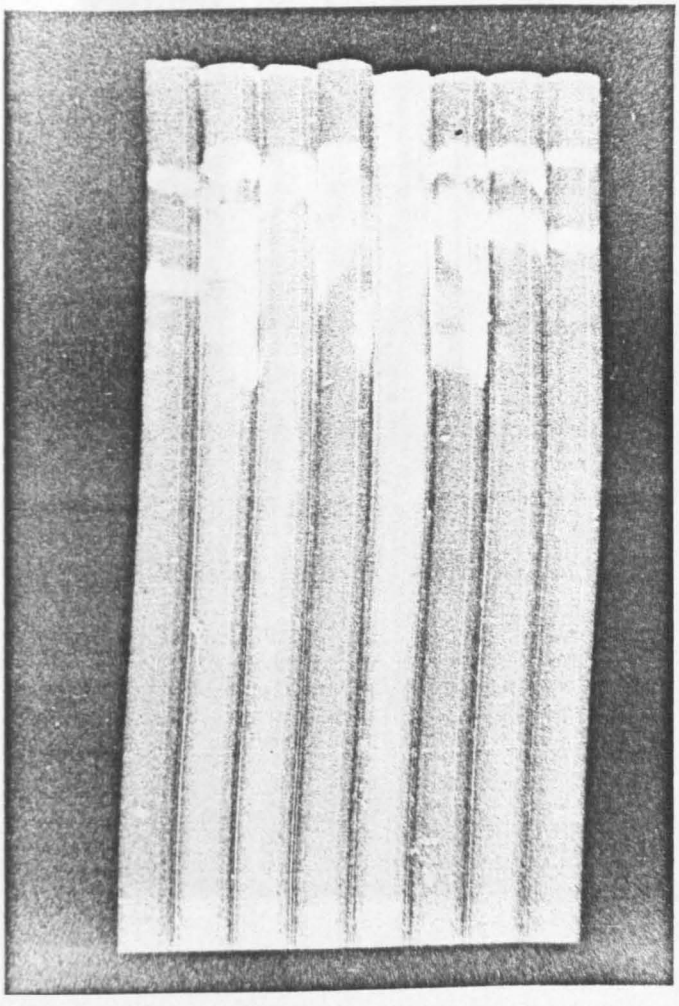


Figure 3.29(a) : Electrophoretic titration of PM2-DNA with 5-iminodaunorubicin, an intercalating drug

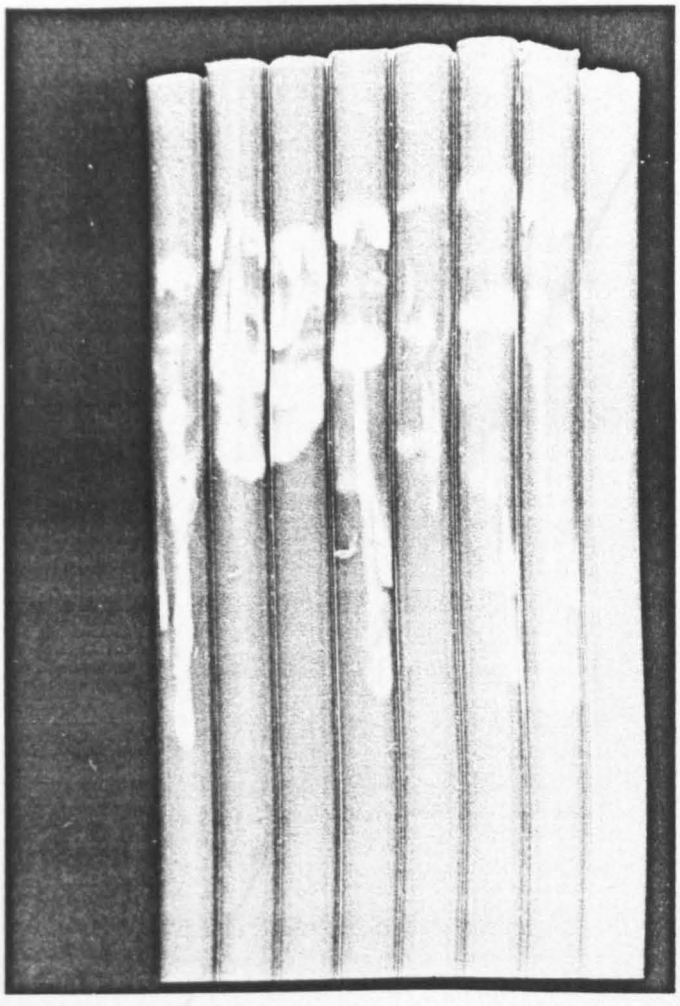


Figure 3.29b: Electrophoretic titration of PM2-DNA with 7-O-methylnogarol, a non-intercalating drug

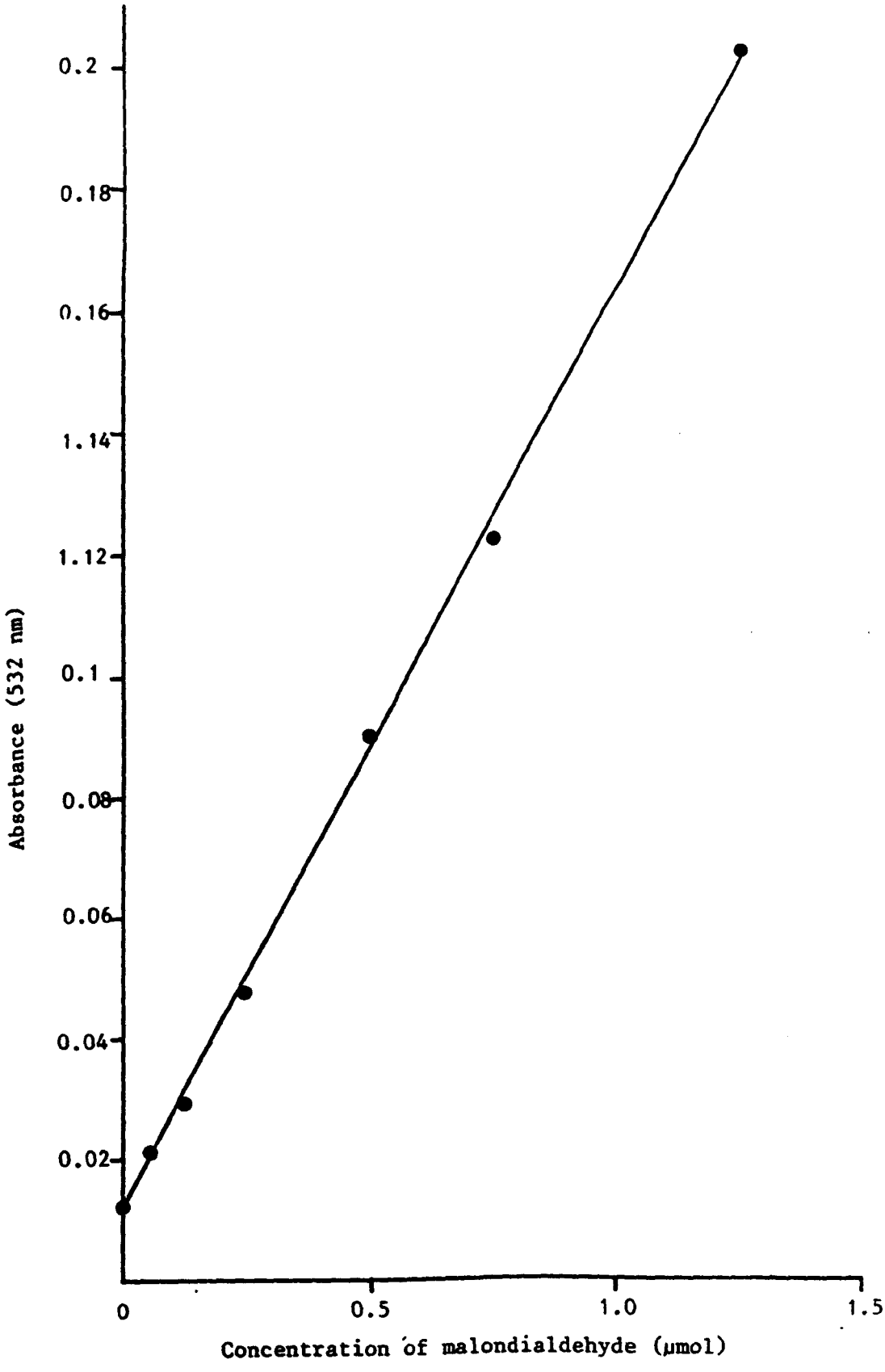


Figure 3.30: Calibration graph for malondialdehyde assay

titration with the non-intercalating drug 7-O-methylnogarol. This drug does not produce the required conformational change in PM2-DNA since the closed circular and nicked circular DNA bands do not co-migrate, or migrate more closely at high drug concentrations.

Table 3.11: Drug concentration required to unwind PM2-DNA (critical concentration) for a series of daunorubicin analogues

Drug	Critical concentration ($\mu g/ml \times 10^2$)
Daunorubicin	24
N-Acetyldaunorubicin	-
N,N-Dibenzyldaunorubicin	-
5-Iminodaunorubicin	10
7-O-Methylnogarol	-
Nogalamycin	40

- means no effect

This group of daunorubicin analogues can, on the basis of these tests, be classified into those drugs which intercalate and those which show little evidence of interaction with DNA. Intercalating drugs reacted similarly to daunorubicin in all of the tests used. These drugs showed marked spectral shifts and fluorescence quenching when titrated with DNA and a large thermal stabilisation of DNA. In addition, these drugs also unwound supercoiled PM2-DNA. Non-intercalating daunorubicin analogues included N-acetyldaunorubicin, 7-O-methylnogarol and N,N-dibenzyldaunorubicin. These showed little evidence of interaction with DNA and gave small spectral shifts little fluorescence quenching and only slight thermal stabilisation of DNA. They also did not cause unwinding of PM2-DNA.

3.5 The role of free radical generation in the trypanocidal activity of daunorubicin

Intercalation of drug into DNA is considered to be the most important mechanism of cytotoxicity among the anthracycline drugs (Zunino et al., 1972), although other mechanisms of cytotoxicity, most notably generation of free radicals via redox cycling of electrons, also may operate. A clinical manifestation of this type of toxicity is seen in the irreversible dose-related cardiotoxicity which occurs after administration of daunorubicin or doxorubicin (Lenaz & Page, 1976). The cytotoxicity of some non-intercalative free radical producing antibiotics is demonstrated by streptonigrin and mitomycin C which are active against various neoplasms (McBride et al., 1966; Crooke and Bradner, 1976) and are thought to act by free radical attack on DNA (Neidle, 1981).

Trypanosomes are thought to be more susceptible than other eukaryotic cells to cellular damage by reactive oxygen species (O_2^-, $\dot{O}H$, H_2O_2) (Meshnick et al., 1977; Boveris and Stopparni, 1977). Protective enzymes such as glutathione peroxidase and catalase, which destroy hydrogen peroxide, are present at low concentrations in trypanosomes (Boveris et al., 1980). Thus compounds such as β-lapachone, which catalyse the generation of superoxide radicals and hydrogen peroxide (Boveris et al., 1978), and haematoporphyrin which catalyses the homolytic cleavage of H_2O_2 to form $OH^{\bullet}$ (Meshnick et al., 1978), are found to be trypanocidal. It may be that anthracycline mediated free radical generation is also important in the trypanocidal activity of these drugs, hence this process has been investigated. Generation of free radical species was monitored indirectly by measuring oxygen consumption (which is increased during production of superoxide radicals and by malondialdehyde production. Malondialdehyde is

produced during lipid peroxidation, and this is stimulated by free radicals. The drugs daunorubicin, 5-iminodaunorubicin and N-acetyl-daunorubicin were chosen for this study as they possess widely different DNA binding affinity and ability to stimulate generation of free radicals. 5-Iminodaunorubicin intercalates into DNA, although not as strongly as daunorubicin; however, N-acetyldaunorubicin interacts very weakly with DNA, and does not intercalate (section 3.4). The ability of these drugs to stimulate generation of free radicals and lipid peroxidation also varies widely. Daunorubicin is known to stimulate lipid peroxidation and generation of superoxide radicals both *in vitro* (Mimnaugh et al., 1982; Lown et al., 1979), and *in vivo* (Patterson et al., 1983). N-Acetyldaunorubicin is likely to possess similar free radical generating properties since both drugs contain identical anthranquinone moieties. In contrast to these drugs, 5-iminodaunorubicin does not stimulate generation of superoxide radicals (Davies et al., 1983), and inhibits lipid peroxidation (Mimnaugh et al., 1982). Lipid peroxidation was estimated by measurement of malondialdehyde using the method described in section 2.5.8 as malondialdehyde production by respiring tissues is accepted as being indicative of lipid peroxidation (Mimnaugh et al., 1982). Standard malondialdehyde solutions were prepared by acid hydrolysis of $^{1,1'}$, $^{3,3'}$ -tetramethoxypropane. A graph of absorbance at 532 nm against malondialdehyde concentration was prepared (Figure 3.30), and an extinction coefficient of 1.51×10^{5} M^{-1} cm^{-1} was calculated from the slope. This value is close to the values of 1.53 and 1.50 M^{-1} cm^{-1} found by Mimnaugh et al. (1982) and Myers et al. (1977), and was used throughout these experiments. The effect of anthracyclines on lipid peroxidation of trypanosomal microsomes was studied *in vitro* and the results are presented in tables 3.12, 3.13 and 3.14.

Table 3.12: Effect of daunorubicin analogues on lipid peroxidation of trypanosomal microsomes in the presence and absence of ferric ions. Incubation time = 60 minutes

Drug	Concentration of malondialdehyde (pmol/mg protein)	Concentration of malondialdehyde in the presence of ferric ion (pmol/mg protein)*
Control	0.6	2.9 ± 0.5
5-Iminodaunorubicin	0.4	3.3 ± 0.7
N-Acetyldaunorubicin	0.5	3.3 ± 0.7
Daunorubicin	0.4	3.4 ± 0.7
Daunorubicin-BSA conjugate (fraction II)	ND	2.7 ± 0.5

* Results are the mean ± standard deviation of three experiments.
ND - not determined.

The amount of trypanosome microsomal lipid peroxidation found in the presence of drug was less than that found for the control value. Facchinetti et al. (1982), who found a similar quenching effect with doxorubicin in rat liver microsomes, suggested that iron was needed as a catalyst. In the present study addition of ferric ion produced a 5-10-fold increase in lipid peroxidation (see Table 3.12). However, the enhancement was independent of the drugs used as, using Student's t test, no significant difference between the results could be found. This suggested that iron-catalysis and not drug was responsible for this effect.

The effect of daunorubicin analogues on lipid peroxidation of liver microsomal and trypanosomal fractions was also investigated after administration of these drugs to trypanosome infected mice *in vivo*. Mice were injected with drug (25 mg/kg) one day after infection with

T. rhodesiense and killed by cervical dislocation in two batches of four mice, four hours and twenty-four hours after drug injection. The livers were removed, pooled and used to prepare microsomal samples. Blood was taken by cardiac puncture and trypanosomes were isolated as described in section 2.6.3. The results are presented in Tables 3.13 and 3.14.

Table 3.13: Effect of daunorubicin analogues on lipid peroxidation of liver microsomes from anthracycline treated mice

Drug *	Time sample taken (hours)	Concentration of malondialdehyde (nmol/mg protein)
Control	24	110
Daunorubicin	4	140
Daunorubicin	24	124
N-Acetyldaunorubicin	4	170
N-Acetyldaunorubicin	24	140
5-Iminodaunorubicin	4	94
5-Iminodaunorubicin	24	90

* 25 mg Kg^{-1} body weight

Liver microsomes isolated from mice treated with daunorubicin or N-acetyldaunorubicin show a slight increase in malondialdehyde content compared with the control mice both after four hours and twenty-four hours. This very slight increase may be due either to the inability of these drugs to provoke significant lipid peroxidation *in vivo* or to metabolism of malondialdehyde. These results are consistent with those of Scheulen et al. (1981) who could not find evidence of lipid peroxidation stimulated by doxurubicin in rats *in vivo* except at a very high dose of 45 mg/kg. However, Myers et al. (1977) demonstrated lipid peroxidation in mice 48 hours after a dose of 15 mg/kg of

doxorubicin, which is twice the dose used in the present study. 5-Iminodaunorubicin showed a slight inhibition of liver microsomal malondialdehyde formation compared to the control value. This is consistent with the results obtained for trypanosomal microsomes (Table 3.12).

The results for trypanosomal homogenate from trypanosome infected, anthracycline treated mice are presented in Table 3.14.

Table 3.14: Effect of daunorubicin analogues on lipid peroxidation of trypanosomal homogenate after isolation of trypanosomes from anthracycline treated mice

Drug	Concentration of malondialdehyde (nmol/mg protein)	Percentage control value
Control	110	100
Daunorubicin	370	336
N-Acetyldaunorubicin	2050	1863
5-Iminodaunorubicin	560	509

All of the drugs tested showed a great enhancement of lipid peroxidation in trypanosomes after 24 hours compared with the livers from trypanosome infected mice. Trypanosomes are known to contain a high concentration of hydrogen peroxide due to a lack of catalase (Meshnick et al., 1977). Hydrogen peroxide can form the highly reactive hydroxyl radical by reaction with the superoxide radical (see section 1.4.2). Since both superoxide and hydroxyl radicals are thought to be involved in the initiation of lipid peroxidation (Kappus and Seis, 1981), trypanosomes may be highly susceptible to lipid peroxidation, and enhancement of these processes in the presence of superoxide generating anthracyclines is likely to occur. N-Acetyldaunorubicin produced the greatest increase in malondialdehyde formation *in vivo*

this being almost six times as great as that caused by daunorubicin. N-Acetyldaunorubicin is more lipophilic than daunorubicin and may be preferentially accumulated in the trypanosomes *in vivo*.

Measurement of oxygen consumption can be used to detect redox cycling by the anthracycline antibiotics since generation of superoxide radicals effectively increases oxygen consumption over the basal rate. This has been demonstrated for daunorubicin and doxorubicin by Goodman and Hochstein (1977), using purified enzymes, who showed that incubation of daunorubicin or doxorubicin with P450 reductase and NADPH causes a great increase in basal rate oxygen consumption. A preliminary experiment was carried out to establish whether enhanced oxygen uptake in the presence of anthracyclines could be demonstrated in trypanosomes *in vitro*. Oxygen consumption of trypanosomes was measured before and after drug addition.

Table 3.15: Oxygen consumption by trypanosomes *in vitro* in the presence of daunorubicin analogues.

Drug	Concentration (mmol)	O_2 consumption (nmol/min/10^6 cells	% Oxygen Consumption (1)	Loss of motility (2)
Daunorubicin	none	11.5	100	++
	0.01	10.9	94.4	
	0.1	4.2	36.7	-
N-Acetyldaunorubicin	none	12.9	100	++
	0.01	13.7	106.5	
	0.1	14.6	112.9	++
5-Iminodaunorubicin	none	12.9	100	++
	0.01	12.4	96.5	
	0.1	11.5	89.3	+-

(1) Expressed as percentage of oxygen consumption in the absence of drug

(2) ++ no loss of motility

+- loss of motility, trypanosomes still motile

- total loss of motility.

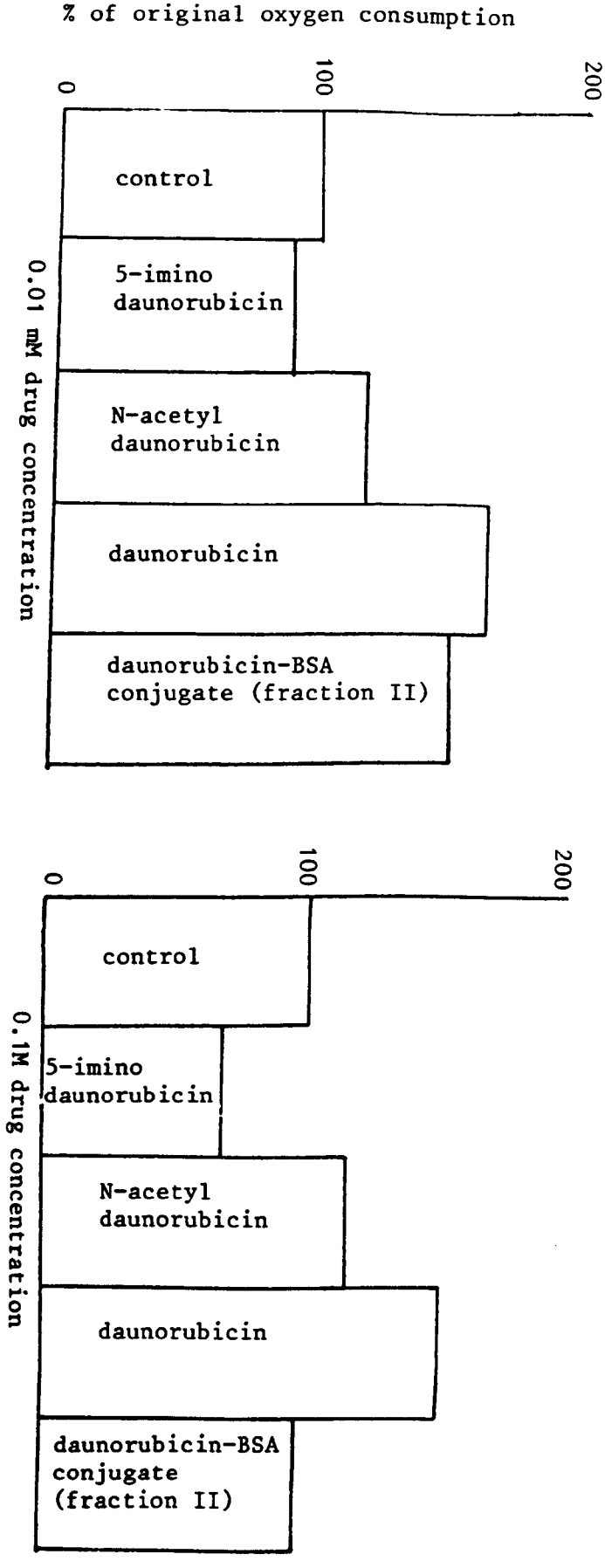


Figure 3.31: Effect of daunorubicin derivatives on oxygen consumption of trypanosomal homogenate

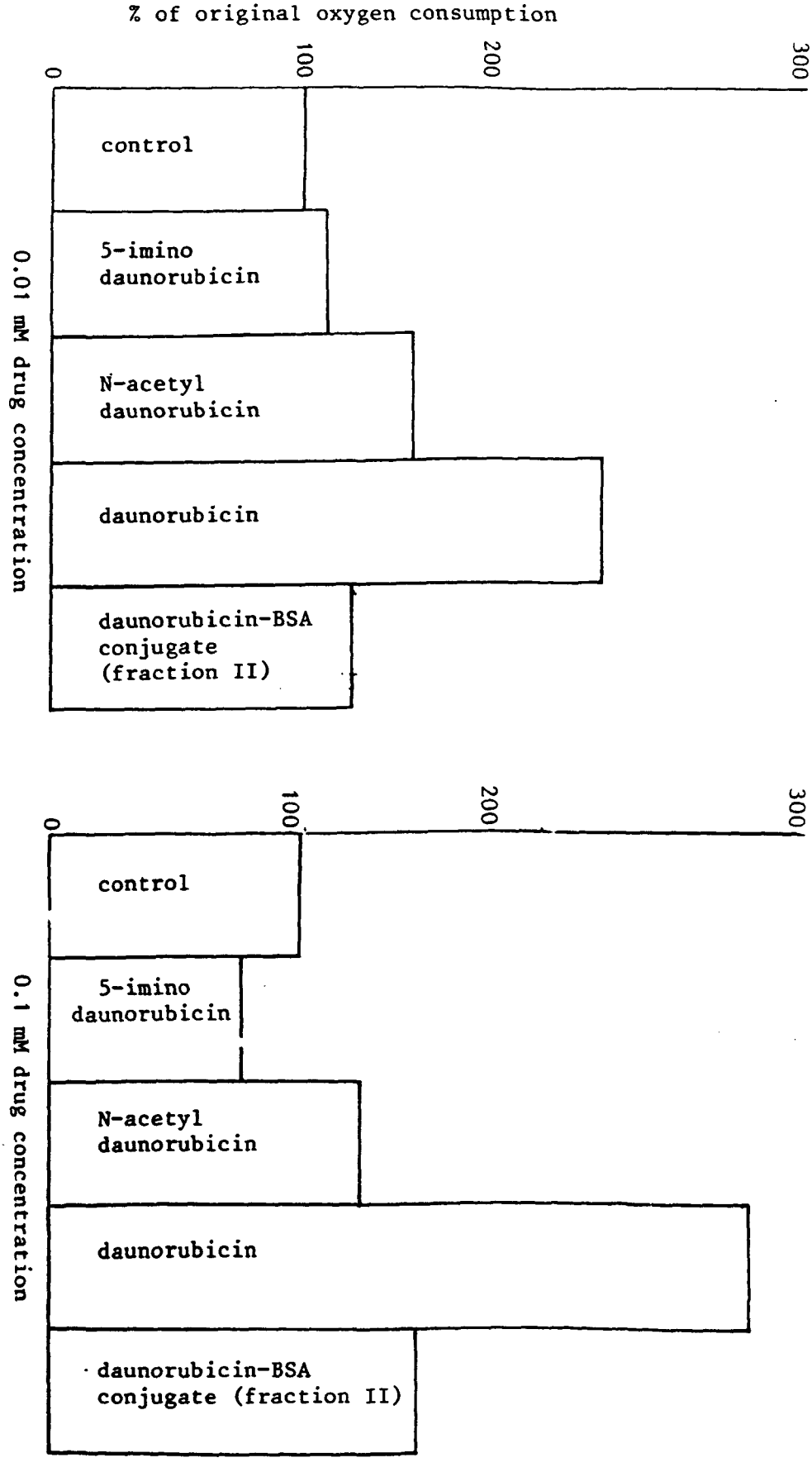


Figure 3.32: Effect of daunorubicin derivatives on oxygen consumption of liver microsomes

Increased oxygen consumption was apparent in the presence of N-acetyldaunorubicin and no loss of motility was observed on microscopic examination. In contrast to this, trypanosomes in the presence of 0.1 mM 5-iminodaunorubicin showed partial loss of motility and in the presence of 0.1 mM daunorubicin showed complete loss of motility. The motility of trypanosomes can be correlated with rates of oxygen consumption and glycolysis (Williamson et al., 1975). With the exception of N-acetyldaunorubicin, which is only weakly trypanocidal (section 3.2), any stimulation of oxygen consumption due to the formation of oxygen radicals appeared to be masked by the decrease in motility.

The effect of daunorubicin analogues on the oxygen consumption of liver microsomes and trypanosomal homogenate was also measured and these results are presented in Figures 3.31 and 3.32. In both experiments the greatest increase in oxygen consumption was seen in the presence of daunorubicin. Precipitation of trypanosomal homogenate occurred at 0.1 mM daunorubicin, which may account for the inhibiting effect seen at this concentration. Daunorubicin-BSA conjugate linked via glutaraldehyde (fraction II) also caused a slight increase in oxygen consumption in trypanosomal homogenate. This may be caused by the presence of free daunorubicin released by enzymic hydrolysis of the drug-glutaraldehyde linkage. 5-Iminodaunorubicin slightly quenches oxygen consumption in liver microsomes and trypanosomal homogenate. The relative inactivity of 5-iminodaunorubicin catalysing production of oxygen radicals has been noted previosuly in heart tissue. Davies et al. (1983) found that daunorubicin stimulated production of superoxide, hydrogen peroxide and hydroxyl radicals in rotenone inhibited beef-heart submitochondrial particles. Stimulation by 5-iminodaunorubicin under the same conditions was less than 7.5% of that produced by doxorubicin.

The anthracycline drugs used in this study may be divided into two groups on the basis of their ability to stimulate lipid peroxidation and oxygen consumption in liver microsomes, which was used as a model system. Daunorubicin and N-acetyldaunorubicin stimulated lipid peroxidation and oxygen consumption in liver microsomes *in vitro* and lipid peroxidation in liver microsomes from drug treated mice. In contrast, 5-iminodaunorubicin inhibited lipid peroxidation and oxygen consumption under identical conditions. In trypanosomes, however, this apparent difference between the drugs was not as marked. Lipid peroxidation of trypanosomal microsomes only occurred in the presence of added ferric ion and under such conditions no significant differences in lipid peroxidation induced by these drugs could be found. All of the drugs caused an increase in oxygen consumption of trypanosomal homogenate, although the increase caused by daunorubicin and N-acetyl-daunorubicin was much greater than that caused by 5-iminodaunorubicin. All three drugs increased lipid peroxidation in trypanosomes isolated from drug treated mice. N-Acetyldaunorubicin produced a much greater increase in lipid peroxidation than either daunorubicin or 5-imino-daunorubicin.

3.6 General Discussion

At the beginning of this study, daunorubicin was known to possess trypanocidal properties, but these could not be translated into *in vivo* activity. In this study a series of daunorubicin analogues and derivatives were synthesised and tested for trypanocidal activity, but only glutaraldehyde linked daunorubicin conjugates were found to be active *in vivo*, when tested against *Trypanosoma rhodesiense* in mice.

The next part of this study concentrated on the factors which affected the activity of these conjugates. Variation in the macromolecule

used produced minor changes in conjugate activity. Neutral, positive and negatively charged macromolecules possessed approximately equal activity, which might reflect a lack of specificity of macromolecule uptake by trypanosomes. Trypanosomes are known to avidly take up proteins by endocytosis.

In contrast, the drug-macromolecule linkage had a major effect on the activity of the conjugates. Apart from glutaraldehyde, all other linking agents produced conjugates which were inactive *in vivo*. Investigation of drug release showed that glutaraldehyde linked daunorubicin-bovine serum albumin conjugate released between 5 and 12% of drug when incubated in pH 7.4 buffer. The amount released depended on the concentration of drug originally linked to the carrier. However, in murine plasma and trypanosomal homogenate approximately 35% of drug was released. Conjugate from which all weakly bound drug had been removed (fraction II) released a further 12% in murine plasma and trypanosomal homogenate. A fraction of the drug made available to trypanosomes *in vivo* by glutaraldehyde linked conjugate is probably enzymically released. It would appear that glutaraldehyde linked conjugate contains at least two bond types. A labile bond which is cleaved in pH 7.4 buffer and a bond which is only cleaved in biological systems. Drug which cannot be cleaved by presumably enzymic hydrolysis may represent a third bond type, or may not be available for enzymic cleavage owing to steric hindrance by the bulky carrier molecule.

The time interval over which drug is released may be an important factor in the activity of daunorubicin conjugates *in vivo*. Glutaraldehyde linked conjugates released drug over a two-hour period. In contrast, hydrazone and oxime linked conjugates released drug very rapidly in pH 7.4 buffer, hence drug will be released before entry

into trypanosomes. The rate of drug release was too rapid to measure in murine plasma and trypanosomal homogenate. Succinyl linked conjugate released about 3% of bound drug during the first thirty minutes of incubation, with none being released thereafter. The period over which drug is released is therefore an important difference between glutaraldehyde linked conjugate and the inactive conjugates tested.

Historically, daunorubicin has been thought to act by interaction with nuclear DNA (Henry, 1976), but in recent years, other cytotoxic mechanisms of action have been proposed. These have included generation of reactive oxygen species (Bachur et al., 1979), and interaction with the cell surface membrane (Tokes et al., 1982). Glutaraldehyde linked daunorubicin conjugate could be postulated to act by any one, or more, of these mechanisms, either by prior release of daunorubicin close to the target site, or as a drug in its own right. Arnon and Sela (1982) showed that a daunorubicin-dextran conjugate was able to reach the nucleus of tumour cells while Lewis (1974) has reported that antibodies which are commonly used as carrier molecules, can be found in the nuclei of tumour cells. In the present study, glutaraldehyde linked daunorubicin-BSA conjugate showed no evidence of interaction with DNA, as judged by spectrophotometric titration and pH shift (Figures 3.24 and 3.25). It also does not markedly stimulate oxygen consumption in trypanosomal homogenate (Figure 3.31) and also inhibits, rather than stimulates, lipid peroxidation in trypanosomal homogenate (Table 3.12), and so is unlikely to act by generation of reactive oxygen species. Glutaraldehyde linked conjugate may be acting on the trypanosome surface membrane and this possibility needs investigating. However, the structurally similar succinyl linked conjugate was inactive *in vivo*. We can thus eliminate the hypothesis that daunorubicin conjugates are

active prior to drug release. In support of this conclusion this study has shown that activity of daunorubicin-BSA conjugates is related to the lability of the linkage. This is supported by a similar study using daunorubicin-BSA conjugate against tumour cells.

Brown et al. (1982) showed that trypanosomes in mice infected with 25 mg/kg daunorubicin were exposed to concentrations 500-fold greater than that required for *in vitro* activity, over a period of six hours. There is evidence that trypanosomes actively excrete daunorubicin (Brown et al., 1982a), and the bloodstream form may be able to eliminate drug more effectively than the *in vitro* form. Since daunorubicin remains in the nucleus of the trypanosome bloodstream form for a short period, it may delay cell division rather than totally inhibit cell division as occurs in the *in vitro* form (Brown et al., 1982a) A conjugate which releases drug over a prolonged period would be expected to provide a continuous intracellular drug concentration, thus inhibiting cell division. Although the breakdown study in trypanosomal homogenate only showed drug release over a two-hour period, this might be considerably longer *in vivo* since metabolic breakdown of the carrier proteins might expose more of the bound drug to hydrolytic enzymes.

Investigation of the mechanism of trypanocidal action of daunorubicin was concentrated on intercalation and free radical damage as indicated by lipid peroxidation. The interaction of several anthracyclines with DNA was studied using a series of tests which measured changes in the properties of the drug and of DNA. Of those tests, a study of the thermal denaturation of DNA in the presence of drug is the most suitable for comparison with trypanocidal properties since it measured a quantitative change in DNA and is applicable to

all drugs. A comparison of change in thermal denaturation temperature with drug activity against *T. rhodesiense in vitro* is shown in Table 3.16. From this table it is clear that only daunorubicin and nogalamycin are active *in vitro*. 5-Iminodaunorubicin, which also binds to DNA, although not as avidly as daunorubicin is, however, inactive. The physical characteristics of a series of daunorubicin derivatives are compared with their activity against *T. rhodesiense* in Table 3.17. The three drugs which exhibit high *in vitro* activity also bind strongly to DNA as shown by their high affinity constants and ΔTm values. *In vitro* activity of these drugs is not directly related to their affinity for DNA, since doxorubicin and 3,4-diepidaunorubicin have higher affinity constants than daunorubicin, yet they are 10^7 times less active in inhibiting trypanosome infectivity *in vitro*. The large differences in potency between these closely related drugs may be considered to be due to pharmacokinetic differences between them, or to two or more different mechanisms of action.

Partition coefficient is an important physicochemical parameter governing the rate of uptake of drugs by cells. Drugs with relatively high partition coefficients are accumulated more rapidly than less lipophilic drugs. This may explain why daunorubicin, which is three times as lipophilic as doxorubicin, is more active *in vitro*, despite having a lower affinity for DNA. Doxorubicin also has a slightly greater inhibiting effect on DNA and RNA synthesis in cell-free systems than daunorubicin (Zunino et al., 1975), but is less active than daunorubicin in tumour cells *in vitro*, the difference being due to greater cellular uptake by daunorubicin (Meriwether and Bachur, 1972). Enhanced uptake of daunorubicin compared with doxorubicin has been observed in tumour cells by Kessel (1974). 4-Demethoxydaunorubicin

Table 3.16: Comparison of change in thermal denaturation temperature (Δ Tm) with *in vitro* inhibition of infectivity and motility in *T. rhodesiense*

Drug	Increase in thermal denaturation temperature (Δ Tm) (°C)	Inhibition of infectivity[1]		Inhibition of motility[1]
		Temporary	Permanent	
Daunorubicin	14.8	11	10	4
Nogalamycin	14.2	> 9	6	4
5-Imino-daunorubicin	9.1	4	3	< 3
N-Acetyl-daunorubicin	2.1	5	< 3	< 3
7-O-Methyl-nogarol	1.75	8	< 3	4
N,N-Dibenzyl-daunorubicin	0.6	< 3	< 3	< 3

(1) Values are the maximum titres ($\log_{10} M^{-1}$) producing the effect; drug with titres < 3 are considered inactive.

Table 3.17: Comparison of activity against *T. rhodesiense* with physical characteristics for a series of daunorubicin derivatives

Drug	Inhibition of infectivity[1]		Inhibition of motility[1]	*In vivo*[4] prolongation of infection	Affinity[2] constants ($M^{-1} \times 10^{-5}$)	Δ Tm (°C)	Partition[3] coefficient	pK_a[5]
	Temporary	Permanent						
Daunorubicin	11	10	4	2.9	13.3	14.8	17.8	8.46
Doxorubucin	10	4	8	1.5	18.8	16.25	6.3	8.34
3,4-Diepi-daunorubicin	10	10	5	1.0	9.7	12.7	-	-
4-Deoxy-daunorubicin	6	3	5	2.0	-	-	-	-
4-Deoxy-doxorubicin	6	3	5	0.5	5.3	8.7	15.0	-
4-Demethoxy-daunorubicin	5	3	5	2.4	9.4	12.8	32.3	7.92
5-Imino-daunorubicin	4	3	3	2.0	-	9.1	-	-

(1) Values are maximum titres ($\log_{10} M^{-1}$) producing the effect; drugs with titres <3 are considered inactive

(2) Plumbridge and Brown, 1979

(3) Cassazza, 1979 n-butanol/tris buffer pH 7.4

(4) Administered as a glutaraldehyde linked BSA conjugate (15 mg/kg)

(5) Zunino et al., 1979

\- Data unknown

gives an inhibition of nucleic acid synthesis in tumour cells equivalent to that of daunorubicin (Zunino et al., 1976) and is more potent than daunorubicin against tumour cells *in vitro* and *in vivo* (Cassazza, 1979). Cellular uptake was fifteen times greater than daunorubicin in L1210 cells *in vitro*, hence high uptake and consequent activity might be expected in trypanosomes. However, 4-demethoxydaunorubicin was only marginally active *in vitro*, hence differences in lipophilicity cannot account for the relative activities of these drugs.

The pK_a is also an important factor affecting cellular uptake of drugs, as it is generally considered that only the neutral form will passively diffuse into the cell. From the pKa data available in Table 3.17, it can be calculated from the Henderson-Hasselbach equation that there will be approximately 8, 10 and 25% of daunorubicin, doxorubicin and 4-demethoxydaunorubicin respectively in the neutral form at pH 7.4. Other drugs in Table 3.17 will have similar pK_a values owing to their similar structure. Since 4-demethoxydaunorubicin is the most unionised drug at physiological pH, the relative lower pK_a should favour uptake of this drug. However, since 4-demethoxy-daunorubicin is only marginally active, pK_a alone is not sufficient to explain differences between drugs.

An alternative explanation of these phenomena is that the anthracycline antibiotics are operating by two or more mechanisms of action and that changes in molecular character modify involvement with one particular mechanism of action, but do not affect (or possibly enhance) involvement with either mechanisms of action. The high activity shown by daunorubicin *in vitro* may therefore be due to a synergistic interaction of DNA binding with some other mechanism of action not shared by 4-demethoxydaunorubicin and other marginally active DNA

binding drugs. Daunorubicin and doxorubicin produce many effects on living cells which may be involved in cytotoxicity. These have included effects on the cell membrane such as transport of small molecules (Dasdia et al., 1979), fluidity (Murphree et al., 1981), covalent binding to phospholipids and proteins (Schwartz et al., 1978) and lipid peroxidation. Other effects which have been noted include inhibition of mitrochondrial activity (Andreini and Beretta, 1977). Some clues to discovering which of the many possible mechanisms of action may be important for a trypanocidal effect may be found from ultrastructural studies.

Many of the actions of the anthracyclines which are not directed specifically to DNA replication and synthesis may be due to lipid peroxidation. Generation of reactive oxygen species and resultant lipid peroxidation was considered a potentially important mechanism of cytotoxicity owing to the vulnerability of trypanosomes to these species (Meshnick et al., 1978). Ultrastructural studies of trypanosomes treated with haem, a compound known to mediate its cytotoxicity by production of hydroxyl radicals, revealed swelling of the endoplasmic reticulum and nuclear envelope (Meshnick et al., 1977). Trypanosomes treated with daunorubicin did not show these features, although there were some indications of membrane damage, most notably the formation of cytoplasmic clefts and autophagic vacuoles (Williamson) et al., 1972). Delain et al. (1982) also noted that daunorubicin caused rearrangement of kinetoplast DNA. However, it is unlikely that anthracycline induced lipid peroxidation is trypanocidal since in this study, N-acetyldaunorubicin, which caused the greatest amount of lipid peroxidation in trypanosomes *in vivo* was active, but

5-iminodaunorubicin, which caused little lipid peroxidation, was active when given as a glutaraldehyde conjugate (see Table 3.10). However, reactive oxygen species do not necessarily produce lipid peroxidation and their attack on cell components other than lipid membranes may be important. Other effects of free radicals, such as depletion of NADPH and glutathione, have not been investigated, although Arrick et al. (1981) have shown that inhibiters of glutathione biosynthesis are trypanocidal.

Bachur et al. (1978) have postulated that anthracycline semiquinone-free radicals may intercalate into DNA and covalently bind to it, or generate reactive oxygen radicals in close proximity to DNA and these may then react with the DNA. Covalent binding of daunorubicin and doxorubicin to DNA has been demonstrated by Sinha and Chignell (1979). This hypothesis might explain the high *in vitro* activity of daunorubicin compared with 5-iminodaunorubicin. Since daunorubicin stimulates oxygen consumption in trypanosomal homogenate and is known to form semiquinone radicals, these might covalently bind to trypanosomal DNA when treated *in vitro*. If these drug-treated parasites are injected into mice, daunorubicin cannot dissociate from the DNA and so permanently inhibit multiplication of the parasite. 5-Iminodaunorubicin does not readily form semiquinone radicals, and also inhibits oxygen consumption in trypanosomal homogenate and is therefore unlikely to react covalently with DNA. If trypanosomes treated with 5-iminodaunorubicin are injected into mice, drug will disassociate from DNA and multiplication will not be inhibited. These differences will not be important if the drugs are administered as a glutaraldehyde conjugate since drug will be available to the trypanosome over a long period of time.

In conclusion, the mechanism of action of daunorubicin was investigated and a relationship between trypanocidal activity and DNA binding ability was revealed. Some DNA binding drugs were not active *in vitro*, suggesting that other mechanisms of action might also be important. Generation of free radical species by the anthracyclines as suggested by monitoring the free radical process lipid peroxidation was investigated, but the results suggest that lipid peroxidation produced by anthracycline generated free radicals is not an important mechanism of cytotoxicity.

This study has also shown that the potent *in vitro* trypanocidal activity of daunorubicin may be reproduced *in vivo* by formation of a macromolecular conjugate. Some of the factors which might affect the activity of these conjugates have been investigated and development of improved conjugate linkages has been attempted.

The use of macromolecular conjugates may, in future, be used for other drugs and in the treatment of other diseases. Targeting of drugs via conjugate formation may enhance the activity of weakly active drugs, and may overcome problems of drug resistance, such as the widespread resistance of malaria parasites to chloroquine. Some work in this direction has recently been published by Trouet et al. (1982) who showed that formation of a labile conjugate of primaquine greatly enhanced its activity against *Plasmodium berghei*.

Daunorubicin is far from being an ideal drug for the treatment of trypanosomiasis on account of its high cost and toxicity. However, mitoxantrone and similar synthetic drugs based on the anthraquinone structure may provide an inexpensive alternative as mitoxantrone has been shown to possess activity against experimental tumours (Cheng et al., 1979), and is less toxic than daunorubicin (Zbinden and Beilstein, 1982).

Labile macromolecular conjugates of the type described in this thesis are important in overcoming problems of drug delivery and low therapeutic index. As they are applicable to so many drugs, it is expected that their use in antiprotozoal chemotherapy will become far more common than it is today.

REFERENCES

ABOLARIN, M. O., EVANS, D. A., ORMEROD, W. E. (1982) Brit. Med. J., 285 (6352), 1380.

ABRAHAM, G. E. AND GROVER, P. K. (1977) in 'Principles of Competitive Protein Binding Assays', p.14o, W.D.Odell and W.H.Daughaday (eds.) Publ. Lippncott, Philadelphia.

ACTON, E. M., JENSON, R. A. and PETERS, J. H. (1981) in 'Anthracycline Anitbiotics in Cancer Chemotherapy', p.204. F. W. Muggia, C. W. Young, S. K. Carter (eds.). Publ. Martinus Nyhoff, Boston.

ADY, P. H. (1965) Regional Ecnomic Atlas: Africa, Publ. Oxford University Press, London.

ANDREINI, G., BERRETTA, C. M. and SONZOONI, O. (1977) Pharmacol. Rev. 9, 155.

ALVING, C. R. (1982) in 'Targeting of Drugs', p. 337, G. Gregoriadis, J. Senior, A. Trouet (eds.). Publ. Plenum Press, N.Y.

ALVING, C. R., STECK, E. A., HANSON, W. L., LOIZEAUX, P. S., CHAPMAN, W. L. jr. and WATTS, V. B. (1978) Life Sci., 22, 1021.

APTED, F. I. C. (1980), Pharmac. Therapeutics, 11, 391.

APTED, F. I. C. (1970) in 'The African Trypanosomiases', p.661. H. W. Mulligan and W. H. Potts (eds.), Publ. Allen and Unwin, London.

APTED, F. I. C. (1957) Trans. Roy. Soc. Trop. Med. Hyg., 51, 75.

APTED, F. I. C. (1953) Trans. Roy. Soc. Trop. Med. Hyg., 41, 387.

ARCAMONE, F., FRANCESCHI, G., PENCO, S., and SELVA, A. (1969) Tet. Lett. 13, 1007.

ARCAMONE, F., FRANCESCHI, G. and PENCO, S. (1974) United States Patent 3, 103, 124.

ARCAMONE, F., FRANCESCHI, G., MINGHETTI, A., PENCO, S., REDAELLI, S., DI MARCO, A., CASSAZZA, A. M., DASDIA, T., DI FRONZO, G., and GIULIANI, F., (1974a) J. Med. Chem. 17, 335.

ARCAMONE, F., (1977) Lloydia, 40, 45.

ARNON, R. and SELA, M. (1982) Immunological Rev., 62, 5.

ARRICK, B. A., GRIFFITH, O. W., and CERAMI, A. (1981) J. Exp. Med., 153, 720.

ASHCROFT, M. T., (1959) Trop. Dis. Bulletin, 56, 1073.

ATKINS, J. (1723) The Navy Surgeon; or Practical System of Surgery with a Dissertation on Cold and Hot Mineral Springs and Physical Observations on the Coast of Guiney. Publ. J. Hodges, London.

AVILA, J. L., BRETANA, A. and AVILA, A. (1979) Am. J. Trop. Med. Hyg., 28, 456.

AXEN, R., PORATH, J. and ERNBACK, S. (1967) Nature (London), 214, 1302.

BACCHI, C. J., (1981) J. Protozool., 28(1), 20.

BACHUR, N. R., GEE, M. V. and FRIEDMAN, R. D. (1982) Cancer Res., 42, 1078.

BACHUR, N. R., GORDON, S. L., GEE, M. V. and KON, H. (1979) Proc. Nat. A Acad. Sci. USA, 76, 954.

BACHUR, N. R., GORDON,SS. L. and GEE, M. V. (1978) Cancer Res., 38, 1745.

BACHUR, N. R., (1976) in 'Cancer Chemotherapy', A. C. Sartorelli (ed.) Publ. American Cancer Society, Washington.

BAURAIN, R., MASQUELIER, M., DEPREZ-de-CAMPANEERE, D. and TROUET, A. (1980) J. Med. Chem., 23, 1171.

BAURAIN, R., DEPREZ-de-CAMPANEERE, D. and TROUET, A. (1979) Cancer Chemother. Pharmacol., 2, 37.

BERDE, C. B., HUDSON, B. S., SIMONI, R. D. and SKLAR, L. A. (1979), J. Biol. Chem., 254, 391.

BERNARD, J., PAUL, R., BOIRON, M., JACQUILOT, C. I. and MARAL, R. (eds.) (1969) 'Rubidomycin'. Publ. Springer-Verlag, Berlin.

BERNSTEIN, A., HURWITZ, E., MARON, R., ARNON, R., SELA, M., and WILCHEK, M., (1978) J. Nat. Canc. Inst. 60,(2), 379.

BITTENCOURT, A. L. (1976) Am. J. Dis. Child., 130, 97.

BIRD, R. P., DRAPER, H. H. and BASRAR, P. K. (1982) Mutation Research 101, 237.

BOVERIS, A., SIES, H., MARTINO, E. E., DOCAMPO, R., TURRENS, J. F., and STOPPANI, A. O. M. (1980) Biochem. J., 188, 643.

BOVERIS, A., DOCAMPO, R., TURRENS, J. F. and STOPPANI, A. O. M. (1978) Biochem. J., 175, 431.

BOVERIS, A. and STOPPANI, A. O. M. (1977) Experentia, 33, 1306.

BROWN, J. E. Ph.D. C. N. A . A. (1983).

BOWMAN, I. B. R., and FLYNN, i. W., (1976) in 'Biology of the Kinetoplastida', 1, 435, W. H. R. Lumsden, D. A. Evans (eds.) Acad. Press. (London) Publ.

BRENER, Z. (1971) Rev. Inst. Med. Trop., 13, 171.

BROHN, F. H., and CLARKSON, A. B. (1978) Acta. Trop., 35, 23.

BROCKMANN, H. and BAUER, K. (1950) Naturwissenschaften, 37, 492.

BROWN, A. W. A., HAWORTH, J. and ZAHAR, A. R. (1976) J. Med. Entomol., 13, 1.

BROWN, J. E., BROWN, J. R. and WILLIAMSON, J. (1982a) J. Pharm. Pharmacol., 34, 236.

BROWN, J. E., PATTERSON, L. H., WILLIAMSON, J. and BROWN, J. R. (1982b) J. Pharm. Pharmacol., 34, 42P.

BROWN, J. R. (1983) in 'Molecular Aspects of Anti-cancer Drug Action' S. Neidle and M. J. Waring (eds.). Pub. Macmillan, N. Y.

BROWN, J. R. (1978) Prog. Med. Chem., 15, 125.

BROWN, K. N., ARMSTRONG, J. A. and VALENTINE, R. C. (1965) Exp. Cell Res., 39, 129.

BRUCE, D. (1895) 'Preliminary report on the tsetse fly disease or nagana in Zululand'. Publ. Bennet and Davis, Durban.

BUNDEGAARD. H. and HANSEN, A. B. (1981) Pharmacy International, 136,

BUXTON, P. A. (1955) 'The Natural History of Tsetse Flies', Mem. London. School of Hygiene and Tropical Medicine. Publ. H. K. Lewis (London).

CALENDI, E., DI MARCO, A., REGGIANI, M., SCARPINTO, B., and VALENTINI, L. (1965) Biochim. Biophys. Acta, 103, 25.

CARL, P. L., CHAKRAVARTY, P. K., KALZENELLENBOGAN, J. A. and WEBER, M. J. (1980). Proc. Nat. Acad. Sci. USA, 77, 2224.

CARTER, S. K. (1975) J. Nat. Cancer Inst., 55, 1265.

CASSINELLI, G. and ORREZI, P. (1963) Giorn. Microbiol., 11, 167.

CASSAZZA, A. M. (1979) Cancer Treat. Rep., 63, 835.

CASTELLANI, A. (1903) Brit. Med. J. Part 1, 1218.

CHENG, C. C., ZBINDEN, R. K. and ZEE-CHENG, Y. (1979). J. Phar. Sci., 68, 393.

CHOE, J. Y., COMBS, A. B., FALKEN, K. (1979) Res. Comm. Chem. Path. Pharmacol., 23, 199.

COUVREUR, P., KANTE, GRISLAIN, L., ROLAND, M. and SPEISER, P. (1982) J. Pharm. Sci., 71, 790.

CRICHTON, R., (1973) Angew. Chem. (Eng. Ed.), 12, 57.

CROOKE, S. T., and BRADNER, W. T. (1976) Cancer Treat. Rev., 3, 121.

DANN, O., FERNBACH, R., PFEIFER, W., DEMANT, E., BERGEN, G., LANG, S., and LARDING, G., (1972) Ann. Chem. 760, 31.

DANN, O., BERGEN, G., DEMANT, E., and VOLZ, G. (1971) Ann. Chem., 749, 68.

DANN, O., HEIKE, E., HAHN, H., MISERRE, H. H., LARDING, G., and ROSSLER, R. (1970) Ann. Chem., 734, 23.

DASDIA, T., DI MARCO, A., GOFFREDI, M., MINGHETTI, A., and NENO, A. (1979) Pharmacol. Res. Commun., 11, 19.

DASGUPTA, S., and DASGUPTA, N. N. (1974) Biochem. Biophys. Acta., 353, 274.

DAVIES, D. A. L. (1974) Cancer Res., 34, 3040.

DAVIES, D. A. L. and NEILL, G. J. O. (1973) Br. J. Cancer, 28 (Suppl. I), 285.

DAVIES, K. J. A., DOROSHOW, J. H. and HOCHSTEIN, P. (1983) FEBS. Lett. 153, 227.

De DUVE, C., De BARSY, T., POOLE, B., TROUET, A., TULKENS, P. and Van HOOFE, F. (1974) Biochem. Pharmacol., 23, 2495.

DELAIN, E., BRACK, C., LACOME, A., and RIOU, G. (1972) in 'Comparative Biochemistry of Parasites', H. Van den Bossche (ed.) Publ. Academic Press, N. Y.

DEPREZ-de-CAMPANEERE, D., MASQUELIER, M., BAURAIN, R., and ROUET, A. (1982) in 'Cell Function and Differentiation' Part A. Publ. Alan R. Liss, N. Y.

DEPREZ-de-CAMPANEERE, D., BAURAIN, R., HUYBRECHTS, M., and TROUET, A. (1979) Cancer Chemother. Pharmacol., 2, 25.

DE RAADT, P. (1976) Trans. Roy. Soc. Trop. Med. Hyg., 70(2), 114.

DE RAADT, P. (1975) in 'Development of Chemotherapeutic Agents for Parasitic Diseases', pp. 105 - 111. M. Marois (ed.). Publ. North-Holland, (Amsterdam).

DI MARCO, A. and ARCAMONE, F. (1975). Arzneim Forsch, 25, 368.

DI MARCO, A., ARCAMONE, F. and ZUNINO, F. (1974) in 'Antibiotics, Vol. III' J. V. Corcoran, F. E. Hahn (eds.). Publ. Springer-Verlag, Berlin.

DI MARCO, A., LENAZ, L., CASSAZZA, A. M. and SCARPINA, B. M. (1972) Cancer Chemother. Pharmacol., 56(2), 153.

DIXON, H., GINGER, C. D. and WILLIAMSON, J. (1971) Comp. Biochem. Physiol., 39(b), 247.

DOCAMPO, R., MORENO, S. N. J., STOPPANI, A..O. M., LEON, W., CRUZ, F. S., VILLALTA, F., and MUNIZ, R. F. A. (1981) Biochem. Pharmacol., 30, 1947.

DOCAMPO, R., CRUZ, F. S., BOVERIS, A., MUNIZ, R. P. A., ESQUIVEL, D. M. S. (1978) Arch. Biochem. Biophys., 186(2), 292.

DOCAMPO, R., De BOISO, J. F., BOVERIS, A. and STOPPANI, A. O. M. (1976) Experentia, 32, 972.

DUGGAN, A. J. (1973) Trop. Doctor, 3, 162.

DUGGAN, A. J. (1970) in 'The African Trypanosomiases', p. 61, H. W. Mulligan and W. H. Potts (eds.), Publ. Allen and Unwin, (London).

Du BOST, M., GUNTER, P., MARAL, R., NINET, L., PINNERTS, PREU D'HOMME, J. and WERNER, G. H. (1963), C. R. Acad. Sci., Paris, 257, 1813.

DUKE, B. O. L. (1978) in 'The Relevance of Parasitology to Human Welfare Today', A. E. R. Taylor, R. Muller (eds.), pp. 1 - 14, Publ. Blackwell.

DUKE, B. O. L. (1981) Brit. Med. J., 283, 961.

DUTTON, J. E. (1902) Thomson. Lab. Rep., 4, 455.

ESPEJO, R. T. and LEBOWITZ, J. (1976), Anal. Biochem., 72, 95.

ESTERBAUER, H. (1982) in 'Free Radicals, Lipid Peroxidation and Cancer'. D. C. H. McBrien and T. F. Slater (eds.). Publ. Acad. Press, london.

ERLANGER, B. F. (1973) Pharmacol. Rev., 25, 271.

FACCHINETTI, T., MUH-ZANGE, M., SALMARA, M., CAVINI, M. and REMMER, H. (1982) Chem. Bio. Interact., 38, 357.

FAIRBAIRN, H. (1953) Ann. Trop. Med. Parasitol., 47, 394.

FAIRLAMB, A. (1982) Trends Biochem. Sci., 7, 249.

FAIRLAMB, A. H. and BOWMAN, I. B. R. (1977) Exp. Parasitol., 43, 353.

FLECK, W., STRAUSS, D., SCHONFELD, C., JUNGSTAN, W., SEELOR, C., and PRAUSER, H. (1972) Antimicrob. Agents Chemother., 1, 385.

FORD, J. (1971) The Role of the Trypanosomiases in African Ecology, Publ. Clarendon Press, Oxford.

FORSSEN, E. A., TODD, J. A., TOKES, Z. A. (1981) Fed. Proc., 40, 644.

FOULKES, J. R. (1981) Brit. Med. J., 283(6300), 1172.

FREIFELDER, D. (1971) J. Mol. Biol., 60, 401.

FREITAS, J. L. P. de, BIANCALANA, A., AMATO NETO, V., NUSSENVEIG, V., SONNTAG, R. and BARRETO, J. G. (1952) Hospital (Rio de J.), 41, 229.

FREIDHEIM, E. A. H. (1949) Am. J. Trop. Med., 29, 173.

FREIDHEIM, E. A. H. (1941) Schweiz Med. Wochenschrift, 71, 116.

FULTON, J. and SPOONER, J. (1956) Biochem. J., 63, 475.

GHOSE, T. and BLAIR, A. H. (1978) J. Nat. Cancer Inst., 61, 657.

GHOSE, T. and NIGARA, S. P. (1972) Cancer, 29, 1398.

GIBSON, W., MEHLITZ, D., LANHAM, S. M. and GODFREY, D. G. (1978) Tropenmed. Parasitol., 29, 129.

GILLES, H. M. (1979) Trans. Roy. Soc. Trop. Med. Hyg., 13, 144.

GOLDSTEIN, A. (1949) Pharmacol. Rev., 1, 102.

GOODFRIEND, T. L., LEVINE, L. and FASHMAN, G. D. (1964) Science, 144, 1344.

GOODMAN, J. and HOCHSTEIN, P. (1977) Biochem. Biophys. Res. Comm., 77, 797.

GOSALVEZ, M., BLANCO, M. F., VIVERO, C., and VALLE, S. F., (1978) Eur. J. Canc., 14(II), 1185.

GREGORIADIS, G., KIRBY, C., LARGE, P., MEEHAN, A. and SENIOR, J. (1982) in 'Targeting of Drugs', G. Gregoriadis, J. Senior and A. Trouet (eds.). Publ. Plenum Press, N. Y.

GREIN, A., SPALLA, C., DI MARCO, A., and CANERAZZI, G. (1963) Giorn. Microbiol., 11, 109.

GROS, L., RINGSDORF, H. and SCHUPP, H. (1981) Angew. Chem. (Eng. Ed.), 20, 305.

GREENBERG, J., CORAL, D., KNUPFER, A. L. and DESHUSSES, J. (1979) Biochem. Biophys. Res. Comm., 88, 1173.

GUIMARES, F. N., SILVA, N. N. da, CLAUSELL, D. T., MELLO, A., RAPORE, L. D., SNELL, T. and RODRIGUEZ, N. (1968) Hospital (Rio de J.), 73, 1767.

GUTTERIDGE, J. M. C. (1979) FEBS Lett., 105, 278.

GUTTERIDGE, W. E., ROSS, J., HARGUDON, H. R. J. and HUDSON, J. E. (1982) Trans. Roy. Soc. Trop. Med. Hyg., 76, 493.

GUTTERIDGE, W. E. (1981) Trans. Roy. Soc. Trop. Med. Hyg., 70(2), 123.

GUTTERIDGE, W. E. and GABORAK, M. (197) Int. J. [illegible]., 79, R43.

HAWKING, F. (1963) in 'Experimental Chemotherapy' 1, 399. Schnitzer and F. Hawking (eds.). Publ. Acad. Press, N. Y.

HAWORTH, J. (1975) in 'Development of chemotherapeutic agents for parasitic diseases', p. 15. M. Marois (ed.). Publ. North-Holland, (Amsterdam).

HENRY, D. W. (1976) in 'Cancer Chemotherapy' p. 15. A. C. Sartorelli (ed.) Publ. American Cancer Society, Washington.

HOARE, C. A. (1970) in 'The Africal Trypanosomiases' pp. 3 - 59. H. W. Mulligan and W. H. Potts (eds.). Publ. Allen and Unwin, (London).

HOARE, C. A. (1964) J. Protozool., II, 200.

HOGBERG, J. BERGSTRAND, A. and JAKOBSON, S. V. (1973) Eur. J. Biochem., 37, 51.

HOWARD, J. E. (1962) in 'La Enfermedad de Chagas Congenita'. Publ. Univ. of Chile, Santiago.

HULHOVEN, R. and DESAGER, J. P. (1976) J. Chromatog., 125, 369.

HURWITZ, E., LEVY, R., MARON, R., WILCHEK, M., ARNON, R. and SELA, M. (1975) Cancer Res., 35, 1175.

ISRAEL, M., PEGG, W. J., WILKINSON, D. M. and GARNICK, M. B. (1978) J. Liq. Chromatog., 146, 795.

JULIANO, R. L. and STAMP, D. (1978) Biochem. Pharmacol., 27, 21.

KANDASWAMY, T. S. and HENDERSON, J. F. (1962) Nature, 195, 85.

KANEKO, Y. (1981) Horm. Metab. Res., 13, 110.

KAPPUS, H. and SIES, H. (1981) Experentia, 15, 1233.

KAYE, S. B., BODER, J. A. and RYMAN, B. E. (1980) Proc. Am. Assoc. Cancer Res., 21, 254.

KERSTEN, W., KERSTEN, H. and SZYBALSKI, W. (1966) Biochemistry, 5, 236.

KESSEL, D. (1979) Biochem. Pharmacol., 28, 3028.

KING, H.,,LOURIE, E. M. and YORKE, W. (1937) Lancet, 233, 1360.

KINNAMON, K. E., STECK, E. A. and RANE, D. S. (1979) Antimicrob. Agents Chemother., 15, 157.

KITAO, T. and HATTORI, K. (1977) Nature, 265, 81.

KOBERLE, F. (1974) in 'Trypanosomiasis and Leishmaniasis', p. 137. Ciba Foundation Symposium 20. Publ. Assoc. Scientific Publishers, (Amsterdam).

KOHN, K. W. and ZWELLING, L. A. (1981) in 'Anthracycline Antibiotics in Cancer Chemotheraphy'. F. M. Muggia, C. W. Young, S. K. Carter (eds.). Publ. Martinus Nyhoff, Boston.

KREBS, H. A. and EGGLESTON, L. V. (1940) Biochem. J., 34, 442.

LAWRENCE, D. J. (1952) Biochem. J., 51, 168.

LEACH, T. M. and ROBERTS, C. J. (1981) Pharm. Ther., 13, 91.

LEGETT-BAILEY, J. (1967) Techniques in Protein Chemistry. Publ. Elsevier, N. Holland, N. Y.

LENAZ, L. and PAGE, J. A. (1976) Cancer Treat. Rev., 3, 11.

LENAZ, L. NECCO, A., DASDIA, T. and DI MARCO, A. (1974) Cancer Chemother. Rep., 58, 769.

LENNAN, L. S. (1964) J. Cell. Comp. Physiol., 84(Suppl. 1), 1.

LESKO, S. A., LORENTZEN, R. J. and T'SO, P. O. P. (1980) Biochemistry, 19, 3023.

LEVI-SCHAFFER, F., BERNSTEIN, A., MESHOVER, A., and ARNON, R. (1982) Cancer Chemother. Rep., 66, 107.

LEWINSOHN, R. (1979) Trans. Roy. Soc. Trop. Med. Hyg., 73,(5), 513.

LEWIS, C. K., PEGRAN, G. D. and EVANS, C. A. (1974) Nature, 247, 463.

LIE, S. O., LIE, K. K. and GLEMSTEIN, A. (1979) Cancer Chemother. Pharmacol., 2, 61.

LIN, J. Y., LI, J. S. and TUNG, T. C. (1981) J. Nat. Cancer Inst., 66. 61.

LOBER, G. and KITTLER, L. (1978) Studia. Biophysica., 73, 25.

LOPES, J. N., CRUZ, F. S., DOCAMPO, R., VASONCELLO, M. E., SAMPAIO, M. C. R., PINTO, A. V. and GILBERT, B. (1978) Ann. Trop. Med. Parasitol., 72, 523.

LOWN, J. W., SIM, S. K., MAJUMBDRAR, K. C. and CHANG, R. Y. (1977) Biochim. Biophys. Res. Comm., 76, 705.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) J. Biol. Chem., 193, 265.

LUND, H. and BJERRUM, J. (1931) Ber., 64, 210.

LYNCH, W. E., SARTIANO, G. P. and GHAFFAR, A. (1980) Am. J. Haematology, 9, 249.

MAAR, E. W. J. de,(1979) Trans. Roy. Soc. Trop. Med. Hyg., 73, 147.

MARSDEN, P. D. (1971) Int. Rev. Trop. Med., 4, 97.

MASQUELIER, M., BAURAIN, R. and TROUET, A. (1980) J. Med. Chem., 23, 1166.

MATHE, G., TRAN BA LOC, P. and BERNARD, J. (1953) C. R. Acad. Sci., 246, 1626.

MAY, P. M., WILLIAMS, G. K. and WILLIAMS, D. R. (1980) Eur. J. Cancer, 16(a), 1275.

MAZZA, S., MANTANA, A., BENITEZ, C. and JANZI, E. Z. (1936) in 'Investigaciones sobre la enfermedad de Chagas'. Publ. MEPRA, (Argentina).

McBRIDGE, T. J., OLESON, J. J. and WOLFF, D. A. (1966) Cancer Res., 26A, 727.

McCORD, J. M. and DAY, E. D. (1978) FEBS Lett., 86, 139.

McGUINESS, J. E., PROCTER, P. H., DEMOPOULOS, H. B., HOKANSON, J. A.[3] and VAN, N. (1979) in 'Proceedings of the Conference on Activated Oxygen and Medicine', Hawaii, Feb. 1979.

MEHLITZ, D., BRINCKMANN, U., HALLER, L. (1981) Tropenme. Parasitol., 32, 129.

MERIWETHER, W. D. and BACHUR, N. R. (1972) Cancer Res., 32, 1137.

MESHNICK, S. R., GRADY, R. W., BLOBSTEIN, S. H. and CERAMI, A. (1978) J. Pharm. Exp. Ther., 207, 1041.

MESHNICK, S. R., CHANG, K. P. and CERAMI, A. (1977) Biochem. Pharmacol., 26, 1923.

MIMNAUGH, E. G., TRUSH, M. A. and GRAM. T. E. (1981a) Biochem. Pharmacol.; 30, 2797.

MIMNAUGH, E. G., TRUSH, M. A., GINSBERG, E., HIROKATA, Y. and GRAM, T. E. (1981b) Toxicol. Appl. Pharmacol., 61, 313.

MINOW, R. A., BENJAMIN, R. S. and GOTTLIEB, J. A. (1975) Cancer Chemother. Rep., 6, 195.

MONG, S., STRONG, J. E., BUSH, J. A. and CROOKE, S. T. (1979) Antimicrob. Agents, Chemother., 16, 398.

MONSIGNY, M., KIEDA, C., ROCHE, A. C. and DELMOTTE, F. (1980) FEBS. Lett., 119, 181.

MORGAN, G. and WALLS, L. P. (1931) J. Chem. Soc., 2447.

MURPHREE, S. A., TRITTON, T. R., SMITH, P. L., and SARTORELLI, A. C. (1981) Biochim. Biophys. Acta, 649, 317.

MYERS, C. E., McGUIRE, W. P., LISS, R. H., IFRIM, I., GROTZINGER, K. and YOUNG, R. C. (1977) Science, 197, 165.

NA'ISA, B. K. (1977) Trans. Roy. Soc. Trop. Med. Hyg., 71, 12.

NAOY, B, RAN. J. and ESDAR, B. (1977) Int. J. Cane, 19, 614.

NEIDLE, S. (1981) in 'Molecular basis of drug action', E. F. Gale, E. Cundliffe, P. E. Reynolds, M. H. R. Climond and M. J. Waring (eds.). Publ. John Wiley & Sons.

NETTLETON, D. E., BALITZ, D. M., DOYLE, T. W., BRADNER, W. T., JOHNSON, D. L., O'HERREN, F. A., SCHREIBE, R. H., COEN, A. B., MOSELEY, J. E. AND MYLLYMAK, R. W. (1980) J. Nat. Prod., 43, 242.

NEIDERER, W. (1970) Experentia, 26, 218.

OLIVIER, L. J. (1974) Z. Parasitenkunde, 45, 197.

OLSON, R. D., McDONALD, T. S., HARBISON, R. D., Van BOXTEL, C. J., BOERTH, R. C., STENIM, A. E. and OATES, J. A. (1977) Fed. Proc.,, 36, 303.

ORMEROD, W. E. (1976) Science, 191(422), 815.

ORMEROD, W. E. (1979) Pharmac. Ther., 6, 1.

PASQUALI-RONCHETTI, I., BINE, A., BATTI, B. De ALOJSIO, G., FORNIERI, C. and VANNINI, V, (1980) Lab. Invest., 42, 457.

PATTERSON, L. H., GANDECHA, B. M. and BROWN, J. R. (1983) Biochem. Biophy. Res. Comm., 110, 399.

PETERS, W. (1978) in 'The Relevance of Parasitology to Human Wefare Today'. A. E. R. Taylor and R. Muller (eds.). Publ. Blackwell Scietific Publishers, Oxford.

PETERS, W. and GILES, H. M. (1977) A Color Atlas of Tropical Medicine and Parasitology. Publ. Wolfe Medical Publications, London.

PIRSON, P., STEIGER, R. F., TROUET, A., GILLET, J. and HERMAN, F. (1979) Trans. Roy. Soc. Med. Hyg., 73, 347.

PLIMMER, H. G. and THOMPSON, J. D. (1908) Proc. Roy. Soc., 80, 1.

PLUMBRIDGE, T. W. and BROWN, J. R. (1979) Biochim. Biophys. Acta, 563, 181.

PLUMBRIDGE, T. W. and BROWN, J. R. (1978) Biochem. Pharmacol., 27, 1881.

POZNANSKY, M. J. and CLELAND, L. G. (1979) in 'Drug delivery systems' R. L. Juliano (ed.). Publ. Oxford University Press, N. Y.

QUIGLEY, G. J., WANG, A. H. J., UGHETTO, G., MAREL, G., Van der BOOM, J. H. (1980) Proc. Nat. Acad. Sci., USA, 77, 7204.

RAHMAN, A., MORE, N. and SCHEIN, P. S. (1982) Cancer Res., 42, 1817.

RAHMAN, A., GUTTERRE, P., RASCHID, S., MATRA, R. L. and SCHEIN, P. S. (1981) Fed. Proc. , 40, 685.

RAJAGOPALAN, S. and SHIFFMAN, M. A. (1974) Guide to simple sanitary measures for the control of enteric diseases. Publ. World Health Organisation, Geneva.

REVIS, N. W. and MARASIC, N. (1978) J. Mol. Cell. Cardial., 10, 945.

RICHARDS, F. M. and KNOWLES, J. R. (1980) J. Mol. Bio., 37, 231.

ROBERT-NICOUD, M. and BACHUR, N. (1983) in 'Targeting of drugs', G. Gregoriadis, T. Senior and A. Trouet (eds.) Publ. Plenum Press, N. Y.

RUBENS, R. D. (1974) Cancer Treat. Rev., 1, 305.

SAMPAIO, M. C. R., PINTO, A. V. and GILBERT, B. (1978) Am. Trop. Med. Parasitol., 72, 523.

SATO, S., LAWIZUMI, M., HANDA, K. and JAMARA, Y. (1977) Gann, 68, 603.

SCHEULEN, M. E., MULIAWAN, H. and KAPPUS, H. (1981) in 'Anthracycline antibiotics in Cancer Chemotherapy', F. M. Muggia, C. W. Young and S. K. Carter (eds.) Publ. Martinus Nyhoff, Boston.

SCHULTZ, C. M. (1976) N. Eng. J. Med., 297, 1259.

SCHWARTZ, H. S., SCHIOPPACASSE, G. and KANTER, P. M. (1978) Antibiol. Chemother., 23, 247.

SCOTT, D. (1970) in 'The African Trypanosomiases', H. Mulligan (ed.) Publ. Allen and Unwin, London. p 615

SHAW, J. J. and LAINSON, R. (1972) Ann. Trop. Med., 66(1).

SHINOZAWA, S., ARAKI, Y. and ODA, T. (1981) Acta Med. Okayama, 35, 395.

SINHA, B. K. and AUST, S. D. (1979) Chemi-Biol. Interact., 28, 301.

SINHA, . . and CHIGNELL, C. F. (1979) Chemi-Biol. Interact., 28, 301.

SKOVSGAARD, T. (1977) Biochem. Pharmacol., 26, 215.

SMITH, T. H., FUJIWARA, A. N., LEE, W. W., WU, H. Y. and HENRY, D. W. (1977) J. Org. Chem., 42, 3653.

SOLLENBERGER, P. Y. and MARTIN, B. R. (1968) in 'The Chemistry of the amino group', S. Patai (ed.) Publ. Interscience Publishers, N. Y.

SOUDJIN, W. (1977) in 'Formulation and preparation of dosage forms', J. Pold (ed.) Publ. Elsevier, N. Holland, Amsterdam.

STEPHEN, L. E. (1970) in 'The African Trypanosomiases', H. Mulligan (ed.) Publ. Allen and Unwin, London.

STEPHEN, J. W. W. and FANTHAM, H. B. (1910) Ann. Trop. Med. Parasitol., 4, 343.

STRAULI, P. and WEISS, L. (1977) Eur. J. Cancer, 13(1), 1.

SUMMERFIELD, F. W. and TAPPEL, A. L. (1981) Anal. Biochem., 111, 77.

THAYER, W. S. (1977) Chem. Bio. Interact., 19, 265.

TOKES, Z. R., ROGERS, K. E. and REMBAUM, A. (1982) Proc. Nat. Acad. Sci., USA, 79, 2026.

TONG, G. L., WU, H. Y., SMITH, T. H. and HENRY, D. W. (1979a) J. Med. Chem., 22, 912.

TONG, G. L., HENRY, D. W. and ACTON, E. M. (1979b) J. Med. Chem., 22, 36.

TONG, G., LEE, W. W., BLACK, D. R. and HENRY, D. W. (1976) J. Med. Chem., 19, 395.

TRITTON, T. R. and YEE, G. (1982) Science, 217, 248.

TROUET, A., MASQUELIER, M., BAURAIN, R. and DEPREZ-de-CAMPANEERE, D. (1982) Proc. Nat. Acad. Sci., USA, 79, 626.

TROUET, A. and DEPREZ-de-CAMPANEERE (1979) Cancer Chemother. Pharmacol., 2, 77.

TROUET, A. (1978) Eur. J. Cancer, 14, 105.

TROUET, A., JADIN, J. M. and VAN HOOFF (1976) in 'Biochemistry of Parasites and Host-Parasite relationship'. H. Van den Bossche (ed.) Publ. Elsevier/N. Holland, Amsterdam.

VAN HIEN, P., KOVACS, K. and MATBOVIES, B. (1975) Enzyme, 19, 1.

VAN HOOF, L. (1947) Trans. Roy. Soc.Trop. Med. Hyg., 40, 728.

VARGA, J. M., ASATO, N., LANDE, S. and LEMER, A. B. (1977) Nature, 267, 56.

VICKERMAN, K. (1976) in 'Biology of the kinetoplastidae', W. H. R. Lumsden and D. A. Evans (eds.) Publ. Acadmic Press, N. Y.

VICKERMAN, K. (1965) Nature (London), 208, 762.

WADDY, B. B. (1970) in 'The African Trypanosomiases', p. 711, H. W. Mulligan and W. H. Potts (eds.) Publ. Allen and Unwin, London.

WARING, M. (1970) J. Mol. Biol., 54, 247.

WARING, M. J. and HENLEY, S. M. (1975) Nucl. Acids Res., 2, 567.

WATKINS, T. I. and WOOLFE, G. (1952) Nature (London), 169, 506.

WETTON, A. F. and AUST, S. D. (1974) Biochim. Biophys. Acta, 373, 197.

WHITAKER, J. R. and GRANUM, P. E. (1980) Anal. Biochem., 109, 156.

WIDDER, K. J., MORRIS, R. M., POORE, G., HOWARD, D. P. and SENYEI, A. E. (1981) Proc. Nat. Acad. Sci., USA, 78, 579.

WILLET, K. C. (1970) in 'The African Trypanosomiases', p. 766. H. W. Mulligan and W. H. Potts (eds.) Publ. Allen and Unwin, (London).

WIESENHAHN, G., VARGA, J. M. and HEARST, J. E. (1981) Nature, 292, 467.

WILLIAMS, D. H. and FLEMING, I. (1973) Spectroscopic methods in organic chemistry. Publ. McGraw Hill, London.

WILLIAMSON, J. (1962) Exp. Parasitol., 12, 274.

WILLIAMSON, J. (1970) in 'The African Trypanosomiases', p. 125. H. W. Mulligan and W. H. Potts (eds.) Publ. Allen and Unwin, (London).

WILLIAMSON, J. (1976) Trans. Roy. Soc. Trop. Med. Hyg., 70(2), 117.

WILLIAMSON, J., MACADAM, R. F. and DIXON, H. (1975) Biochem. Pharmacol., 24, 147.

WILLIAMSON, J, McLAREN, D. J. and BROWN, J. R. (1983) Cell Biol. Int. Rep., 7, 997.

WILLIAMSON, J. and SCOTT-FINNIGAN, T. J. (1975) Trans. Roy. Soc. Trop. Med. Hyg., 69, 1.

WILLIAMSON, J. and SCOTT-FINNIGAN, T. J. (1978) Antimicrob. Agents Chemother., 13, 735.

WILLIAMSON, J., SCOTT-FINNIGAN, T. J., HARDMAN, M. A., BROWN, J. R. (1981) Nature, 292, 466.

WILLIAMSON, J. (1979) Pharmacol. Ther., 7(3), 445.

WILSON, S. G., MORRIS, K. R. S., LEWIS, I. J. (1963) Bull. W. H. O., 28, 595.

WINTERBOURNE, C. C. (1981) FEBS Lett., 136, 89.

WORLD HEALTH ORGANISATION (1979) Report of the second meeting of the Scientific Working Group on Leishmaniasis, UNDP/World Bank/WHO Soecial Programme for Research and Training in Tropical Diseases.

WORLD HEALTH ORGANISATION (1967) Technical Report Series 359.

WRAGG, W. R., WASHBOURNE, K., BREICIN, K. N. and HILL, J. (1958) Nature, 182, 1005.

YAMAMOTO, K., ACTON, E. M. and HENRY, D. W. (1972) J. Med. Chem., 15, 872.

YORKE, W. (1929) Ann. Trop. Med. Parasitol. 23, 501

ZAHARKO, O. S., PRZYBYLS, M. and OLIVERIO, V. T. (1979) Meth. Cancer Res., 16, 347.

ZBINDEN, G. and BEILSTEIN, A. K. (1982) Toxicol. Lett., 11, 289.

ZELEDON, R. and RABINOVICH, J. E. (1981) Ann. Rev. Entomol., 26, 101.

ZELEDON, R. (1974) in 'Trypanosomiasis and Leishmaniasis', p. 51. Ciba Foundation Symposium 20. Publ. Assoc. Scientific Publishers, (Amsterdam).

ZUNINO, F., GAMBETTA, R., VIGEVANI PENCO, S., GERONI, C. and DI MARCO, A. (1981) Tumori, 67, 521.

ZUNINO, F., DI MARCO, A., ZACCHARA, A., and GAMBETTA, R. A. (1974) Biochim. Biophys. Acta, 607(2), 206.

ZUNINO, F., GAMBETTA, R. A., DI MARCO, A., LUOSIC, G. and ZACCARA, A. (1976) Biochim. Biophys. Res. Comm., 69, 744.

ZUNINO, F., GAMBETTA, R. A., and DI MARCO, A. (1975) Biochem. Pharmacol., 24, 309.

ZUNINO, F., GAMBETTA, R. A., DI MARCO, A. and ZACCARA, A. (1972) Biochim. Biophys. Acta, 277, 489.

ZWELLING, L. A., KERRIGAN, D. and MICHAELS, S. (1982) Cancer Res., 42, 2687.

APPENDIX

Preparation of buffers and physiological media

(a) Phosphate buffered saline (P.B.S.) pH 7.4 contains:

Na_2HPO_4 - 66 mM (9.37g/L)

KH_2PO_4 - 8.7 mM (1.18g/L)

NaCl - 150 mM (8.766g/L=

(b) Tris buffer A pH 7.4 contains:

NaCl - 50 mM (2.92g/L)

$NH_2C(CH_2OH)_3$ - (0.969g/L)

neutralised to pH 7.4 with hydrochloric acid

(c) Tris buffer B pH 7.4 contains:

$NH_2C(CH_2OH)_3$ - 50 mM (6.05g/L)

EDTA - 2 mM (0.584g/L)

neutralised to pH 7.4 with hydrochloric acid

(d) Citrate saline contains:

NaCl - 145 mM (8.5g/L)

Sodium Citrate - 58 mM (15g/L)

The solution was filtered and then sterilised in an autoclave.

(e) Kreb's phosphate saline contains:

0.9% NaCl - 1L

1.15% KCl - 40 ml

3.84% $MgSO_4.7H_2O$ - 10 ml

* 0.1M phosphate buffer - 300 ml

* Buffer contains:

Na_2HPO_4 - 100 mM (14.2g/L)

HCl (1M) - 20 ml

(f) Kreb's glucose contains:

Glucose - 111 mM (20g/L) in Kreb's phosphate saline solution.

(g) NADPH generating solution contains:

NADP (1.62 mg/ml)

Glucose-6-phosphate (2.61 mg/ml)

Glucose-6-phosphate dehydrogenase - 1.5 units/ml

$MgCl_2$ (1M) - 10 µl

in 1 ml of 0.1M phosphate buffer pH 7.4*

* 0.1M phosphate buffer contains

0.2M disodium hydrogen phosphate - 40.5 ml

0.2M sodium dihydrogenphosphate - 9.5 ml

made up to 100 ml with water.

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