PHOTODYNAMIC THERAPY WITH HAEMATOPORPHYRIN DERIVATIVE

A thesis submitted to the University of Adelaide as a requirement for the degree of Doctor of Philosophy.

by

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ABSTRACT

The photodynamic activity of haematoporphyrin derivative (HPD) is used to treat malignant tumours in man. HPD is a complex mixture of porphyrins, some of which may be more phototoxic than others. The only known side effects are skin photosensitivity and damage to structures adjacent to the treated tumours.

The aim of this thesis was to investigate methods of improving the specificity and efficacy of the photodynamic destruction of tumours by:

(1). Identifying the most photoactive porphyrins in HPD both *in vitro* and *in vivo*.

(2). Examining the potential improvement in the specificity of uptake of porphyrins into tumours by coupling porphyrins to tumour-specific antibodies.

(3). Modulating the photodynamic response by the concurrent administration of other pharmacological agents.

Relative activities of the components of HPD were examined *in vitro* using ^{51}Cr release as a measure of photodynamic damage. The most photoactive porphyrins were HPD, HPD aggregate, aggregated hydroxyethyl vinyl deuteroporphyrin (HVD) and protoporphyrin (PP). Less aggregated HPD and HVD were less active and haematoporphyrin (HP) was inactive. Porphyrin fluorescence in the cells corresponded with photoactivity, suggesting uptake was vital for photoactivity *in vitro*.

The photoactivity *in vivo* of some of the components of HPD was examined using a transplantable tumour model in mice. HPD, HPD aggregate and the commercial product, Photofrin II showed equal phototoxicity. Non-aggregated HPD, PP and HP were

inactive. Uptake of porphyrins (measured by fluorescence) in tumours and skin photosensitizing ability of porphyrins corresponded with photoactivity.

When HP was covalently coupled to polyvalent rabbit antihuman immunoglobulin, the photosensitizing activity of HP was retained but the antibody specificity of the HP-antibody conjugate for B lymphocytes was destroyed. This suggests that there are serious problems to be overcome if this approach is to be useful.

Adriamycin and methotrexate potentiated the response of Lewis lung carcinoma to photodynamic therapy (PDT). Cyclophosphamide, vincristine, thiotepa or 5-fluorouracil all had no effect. The increase in efficacy of PDT after administration of Adriamycin or methotrexate corresponded with an increase in porphyrin fluorescence in the tumours.

Although Adriamycin potentiated PDT in vivo, it inhibited photodynamic damage in vitro. Fluorescence microscopy indicated that Adriamycin inhibited HPD uptake.

The effect of glucocorticoids on the efficacy of PDT was also tested. Methylprednisolone inhibited uptake of HPD with a corresponding reduction in efficiency of PDT. By contrast, administration of glucocorticoid after PDT enhanced the therapeutic response by reducing the rate of recurrence. These results may have considerable clinical significance.

The influence of vasoactive drugs on PDT was also examined. Verapamil potentiated PDT, with a corresponding increase in the intensity of fluorescence in the tumours. The vasoconstricting agent noradrenaline, when administered concurrently with HPD, inhibited PDT by reducing uptake of HPD into the tumours. Noradrenaline administered with irradiation

had no effect. The vasodilating agents propranolol and hydralazine had no effect on the efficacy of PDT.

STATEMENT

(a). This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge and belief contains no material previously published or written by any other person, except where due reference is made in the text.

(b). I consent to this thesis being made available for photocopying and loan.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Photodynamic therapy (PDT) has been recently introduced for the diagnosis and treatment of malignant tumours in man [for recent reviews see Dougherty, 1984a; Dougherty et al., 1985; Kessel, 1984a; 1984b]. A photosensitizing and tumour-localizing drug, haematoporphyrin derivative (HPD) is administered intravenously to the patient, followed by exposure of the tumour to red light. Photoactivation of HPD in the tumour causes a phototoxic reaction which results in necrosis of the tumour. The nature of these interactions will be discussed below.

(a). Historical background

The capacity of phototoxic reactions to cause cell death was demonstrated by Raab [1900], who incubated paramecia in acridine orange and observed cell death after exposure to sunlight. Tappenier and Jesionek [1903] suggested the possibility of treating tumours by a photodynamic reaction. They attempted to treat human skin tumours with eosin and light.

The phototoxic activity of haematoporphyrin (HP) was first described by Hausmann [Hausmann, 1908; 1914]. He incubated paramecia or red blood cells with HP and showed that subsequent exposure to light resulted in cell death. He also injected HP (2mg) into mice and observed a fatal phototoxic reaction. Meyer-Betz [1913] developed severe cutaneous photosensitivity after injecting himself intravenously with 200mg HP. Cutaneous photosensitivity was also observed in dogs

after an intravenous injection of 60mg/kg HP [Rask and Howell, 1928].

Policard [1924] detected spontaneous red fluorescence in tumours, but not in normal tissue when he illuminated the tissues with a Wood's lamp. He attributed this fluorescence to endogenous porphyrins produced by bacteria in the tumours.

The capacity of HP to localise specifically in tumours was first investigated by Auler and Banzer [1942]. Crude HP was injected into tumour-bearing rats and preferential uptake in tumours was demonstrated by fluorescence.

(b). Porphyrin fluorescence for detection of tumours

The diagnostic potential of the localization and fluorescence of porphyrins in tumours was investigated by Figge [Figge et al., 1948; Figge and Peck, 1953; Figge et al., 1956]. Red fluorescence was observed in tumours, lymph nodes, traumatised tissue and embryos in mice after irradiation with near ultraviolet light. There was greater intensity of fluorescence in necrotic areas of tumours. It was concluded that rapidly growing tissues had an affinity for HP, roughly proportional to the mitotic index.

Rasmussen-Taxdal showed selective red fluorescence of HP in tumours and lymph nodes and suggested using fluorescence to delineate tumours during surgery [Rasmussen-Taxdal et al., 1955]. The selective uptake of HP in rat tumour but not in muscle was also shown by measuring porphyrins extracted from tissues [Winkelman and Rasmussen-Taxdal, 1960].

The efficacy of detection of tumours by fluorescence of porphyrins was improved by the development of haematoporphyrin derivative (HPD) by Lipson [Lipson & Baldes 1960a; Lipson et

al., 1961a]. A smaller dose of HPD was required to obtain good fluorescence in the tumours and, in contrast to HP, there was only minimal fluorescence in other tissues, indicating improved tumour selectivity. There was good correlation between red fluorescence of porphyrins in small lung cancers and histologically proven tumour [Lipson et al., 1961b; Lipson et al., 1964a].

Since the first studies of Lipson, a number of workers have investigated the use of HPD fluorescence for the diagnosis of tumours. Efficacy of detection of fluorescence has been greatly improved in recent years by the development of new technologies. Lasers have been used as the exciting light source, with delivery of the light through fibreoptic bronchoscopes and better detection of weak fluorescence from small tumours.

New methods have been developed for the detection of carcinoma in situ by fluorescence bronchoscopy [Profio et al., 1979; Doiron et al., 1979; King et al., 1982]. There was good correlation between fluorescence and histologically proven tumour when patients with sputum positive for malignant cells but radiographically normal were examined. Kinsey and Cortese [Kinsey et al., 1978; Cortese et al., 1979; 1982] reported accurate localization of tumours by fluorescence bronchoscopy, although they also saw fluorescence in areas of moderate and marked squamous cell atypia. Hayata reported good correlation between fluorescence and histologically proven tumours. Positive fluorescence was also detected in areas of squamous metaplasia [Hayata et al., 1982a; 1982b]. Occasional false negative results were reported which were attributed to tumours being occluded by blood, necrotic tissue or normal

mucosa. Thus it appears that fluorescence bronchoscopy with HPD may be an important technique for the detection of small occult lung tumours.

Fluorescence has also been used to detect carcinoma in situ in the bladder. Benson et al. [1982] administered 2.5mg/kg HPD to patients 2-48h before cystectomy. The resected bladder was then examined under a fluorescent light and good correlation was observed between red fluorescence and the presence of neoplastic or dysplastic cells. Recent advances in instrumentation have allowed detection of low levels of HPD fluorescence in tumours. This should lead to more effective detection of multifocal carcinoma in situ in the bladder [Lin et al., 1984].

(c). Use of HPD in photodynamic therapy of tumours

(i). Animal studies

An early report of the application of the photodynamic activity of porphyrins for treatment of tumours was by Diamond who transplanted glioma cells subcutaneously in rats. Treatment with HP and white light resulted in destruction of the tumours [Diamond et al., 1972].

The photodynamic destruction of tumours using HPD was reported by Dougherty et al. [1975]. All animal tumours tested responded to treatment with HPD and light. The greatest uptake of HPD in the tumour was at 24h, with a tumour:liver ratio of approximately 4:1. Selective HPD uptake and photodynamic destruction of human bladder carcinoma grown in immunosupressed mice was demonstrated by Kelly and coworkers. Normal bladder tissue was not damaged and they suggested photodynamic therapy with HPD could be used to treat human transitional cell

carcinoma of the bladder [Kelly et al., 1975].

Animal models of human disease have been developed to assess PDT as a therapeutic modality and to develop methods for treatment. Squamous cell carcinoma was induced in the bronchus of dogs by injection of 20-methylcholanthrene and was then treated by PDT. Complete response, judged histologically, was seen with all treated tumours with minimal damage to the normal bronchus [Hayata et al., 1983].

Animal models of bladder carcinoma in mice and rats [Bellnier et al., 1985] and in dogs [Nseyo et al., 1985a; 1985b] have been developed. Nseyo investigated methods for irradiating the whole canine bladder via a cystoscope and using a bulb-type fibreoptic tip to produce an isotropic light pattern. With moderate light doses, there was some inflammation of the epithelium but minimal damage to the bladder muscle. However, higher light doses caused shrinkage and subsequent necrosis of the bladder [Nseyo et al., 1985b]. The use of dispersing medium to allow good light scatter through the bladder has been investigated by Jocham et al. [1984]. They used fat emulsions (Intralipid) and demonstrated isotropic light distribution and minimal loss of light energy (10-50%). Treatment of rabbit bladder containing transplanted Brown-Pearce tumour resulted in complete destruction of tumours but with minimal damage to normal mucosa.

Effective treatment of solid tumours has been demonstrated in two rat prostate tumour models using interstitial delivery of light [McPhee et al., 1984]. An ovarian ascitic tumour in a mouse was treated by intraperitoneal irradiation via a fibreoptic [Tochner et al., 1985]. The ascites responded well

to PDT with a decrease in weight and abdominal size, but destruction of tumour cells was incomplete. However, once technical problems with light delivery are overcome and tolerance of normal tissue to PDT determined, intraperitoneal PDT may be a potential treatment for ovarian tumours.

Animal models of human retinoblastoma and amelanotic melanoma have been developed to examine tumour responses, treatment techniques, and responses of normal ocular tissue to PDT. Benedict et al. [1980] showed that human retinoblastoma transplanted in the eye of a nude mouse was responsive to PDT. Amelanotic melanoma in the eye of a rabbit responded to PDT but uptake of HPD and photodamage to vascularised normal structures was also observed [Gomer et al., 1983; 1984a; 1985a]. These results were confirmed by Franken et al. [1985]. Provided care is taken with light delivery and the dose rate carefully monitored, PDT may be safely used in the eye with good tumour destruction.

PDT has also been used for veterinary treatment of primary tumours in cats and dogs [Dougherty et al., 1981]. Good responses were obtained in a range of tumours, resulting in long term control and possibly cure. Similar results were obtained by Cheli et al. [1984], who also treated tumours in cats and dogs. PDT was suggested as an alternative to surgery in some cases in veterinary oncology.

(ii). Clinical studies

The first clinical use of HPD-PDT was in 1976 by Kelly and Snell, who treated a patient with superficial bladder carcinoma. They observed necrosis only in the irradiated area [Kelly and Snell, 1976]. A major clinical assessment of PDT for cancer therapy was carried out by Dougherty and coworkers at

the Roswell Park Memorial Institute [Dougherty et al., 1978]. They treated a series of cutaneous and subcutaneous tumours using an incandescent Xenon arc lamp with red filters (600-700nm) and obtained excellent therapeutic responses. A further study [Dougherty et al., 1979] demonstrated the efficacy of PDT in controlling breast cancer metastatic to the chest wall with minimal damage to normal skin.

The group from The Queen Elizabeth Hospital published a report in 1980, which confirmed the above results on the efficacy of PDT for treating cutaneous and subcutaneous tumours [Forbes et al., 1980]. Good tumour necrosis was observed, with only two cases of highly pigmented metastatic melanoma and one case of chondrosarcoma failing to respond to PDT. Effective treatment by PDT of metastatic breast cancer and other cutaneous malignancies was also reported by Dahlman et al. [1983] and Konaka and Ono [1983].

Gynaecological tumours also respond well to PDT. Ward et al., [1982] treated tumours in the vagina by interstitial placement of fibreoptics and obtained destruction of tumours with reepithelialisation in 3 out of 5 cases. Soma et al. [1982] and Rettenmaier et al. [1984] also reported good responses of gynaecological tumours to PDT.

Treatment of bronchial carcinoma may be a very important application of PDT. By using a flexible bronchoscope, light may be directed through an optical fibre into the tumour. Major advances have been made by Hayata's group in Tokyo, who demonstrated good therapeutic results in the treatment of bronchial carcinoma [Hayata et al., 1982a; 1982c]. PDT was used for cure of early stage tumours [Hayata et al., 1984],

opening of blocked bronchi in advanced cases, converting inoperable cases to operable, and to reduce the extent of resection [Kato et al., 1985].

Treating endobronchial tumours with PDT has also been investigated by Balchum et al. [1984]. They reported good therapeutic responses with full opening of blocked bronchi, re-expansion of collapsed lungs and reduction of symptoms. They emphasized the necessity for bronchoscopy 3 days after treatment to remove exudate and necrotic tumour, thus avoiding obstruction and pneumonia. Cortese and Kinsey [1984] also demonstrated the potential efficacy of PDT in the treatment of bronchial tumours. However they reported two cases of fatal haemoptysis in patients where the tumour extended distally through the bronchial wall. Other reports confirming the efficacy of PDT in treating bronchial carcinoma are from Beijing [Li et al., 1984], University of California, Irvine [Wile et al., 1984a] and Roswell Park Memorial Institute [Vincent et al., 1984]. HPD dose (2.5-3.0mg/kg) and effective light doses (approximately 200J/cm for interstitial placement) have been defined. Light delivery methods to obtain optimum tumour destruction have also been developed. Potential complications such as obstruction by mucous exudate and late haemorrhage due to erosion of underlying blood vessels have been recognised. Currently a multicentre trial comparing PDT plus radiotherapy to PDT is being organised as a major step in the clinical acceptance of PDT.

The experience of treating carcinoma of the bronchus at The Queen Elizabeth Hospital has been in agreement with the results of other workers. Effective destruction of tumours has been observed in most patients with remissions of up to 1 year

and also the conversion of inoperable to operable in one patient. Mucous plug and debris is removed at bronchoscopy 72h after irradiation. There have been two cases of tracheo-oesophageal fistula which resulted from necrosis of tumour eroding through the bronchial wall.

PDT may also play an important role in the treatment of bladder carcinoma. Good localization of HPD to neoplastic and dysplastic tissue in the bladder has been shown [Benson et al., 1982]. Treatment of 4 patients with carcinoma in situ resulted in disappearance of tumour [Benson et al., 1983]. Hisazumi also eradicated small tumours (<2cm) with PDT [Hisazumi et al., 1983]. Tsuchiya et al. [1983] also obtained excellent tumour responses, with 6 out of 8 patients remaining disease free up to 18 months later. Future developments such as the use of microlenses on the fibreoptic tip, or a diffusing medium to allow irradiation of the whole bladder, will improve the efficacy of treatment.

PDT is being assessed for the treatment of head and neck cancers [Taketa and Imakiire, 1983; Ossoff et al., 1984; Wile et al., 1984b; Schuller et al., 1985; Carruth and McKenzie, 1985] and oesophageal carcinoma [Aida and Hirashima, 1983; McCaughan et al., 1984; Morstyn, unpublished results, 1985]. Results appear encouraging, with good tumour responses and clinically useful palliation. Optimum light dosimetry and methods of light delivery need further investigation, but PDT may become an important method of treatment for these tumours.

Treatment of intraocular tumours by PDT has also been investigated. Bruce [1984] obtained a significant reduction in tumour volume in choroidal malignant melanoma. Complete

obliteration of blood flow in the treated area was also noted. There was some reduction in visual acuity, particularly when the tumour was near the macula. Ocular melanoma was treated with PDT with good results and disappearance of tumour in 1 out of 6 patients (1 year follow up) [Tse et al., 1984]. However they experienced problems with transient iritis, and reported two cases of retinal detachment and two of neovascular glaucoma. This raises doubts as to the safety of PDT in the eye. Preclinical studies on animal models are currently defining safe light doses and methods of irradiation to allow PDT to be used effectively in the eye.

A potentially very important application of PDT is in the treatment of malignant brain tumours. Currently, the two year survival of patients with anaplastic gliomas after surgery and radiotherapy is 7.5% [Salcman, 1980]. Addition of an extra treatment modality may be very useful. McCulloch treated patients with high grade malignant gliomas and metastatic tumours at craniotomy. The bulk of the tumour was removed and the cavity irradiated. The treatment was well tolerated, with the major side effect being oedema of the irradiated brain. Results were encouraging, with 2/8 patients with grade 4 astrocytoma alive and well 4 years later [McCulloch et al., 1984]. Laws et al. [1981] studied the feasibility of treating brain tumours by PDT. Necrosis of human glioma was demonstrated after irradiation of the brain at craniotomy [Perria et al., 1980; Perria, 1981]. Kaye [1985, personal communication] has treated patients with glioma by irradiating the tumour bed at craniotomy after filling the cavity with a light dispersing medium. Thus treatment of brain tumour is feasible and tumour necrosis has been demonstrated, but clinical trials are needed

to determine if survival is improved by the addition of PDT to the conventional treatments.

In summary, PDT is effective in causing necrosis of tumours. It is a local treatment and limited light penetration through tissues means large tumours can not be treated effectively. Patients are not suitable for PDT where tumour necrosis would cause additional problems, for example, haemorrhage due to tumour invading large blood vessels or extensive cutaneous ulceration. Thus PDT, when used as the sole treatment, appears to be most successful in treating smaller tumours and not in debulking large tumour masses. Cure of small early tumours is possible. PDT may also be useful where other modalities are not suitable or have failed. Indications for PDT need to be defined and examined in carefully designed clinical trials. Methods of light application also need improvement, particularly the design of fibreoptic tips. The major side effect of skin photosensitivity could be reduced by development of appropriate sunscreens. A recent report of a topical HPD preparation to be applied directly to cutaneous tumours rather than by systemic injection may also be potentially useful [McCullough et al., 1983].

(d.) Mechanisms of uptake of HPD

(i). Uptake and retention of HPD in isolated cells

The mechanisms of uptake of HPD into cells and the sites of intracellular localization are still unclear. HPD uptake could be by passive diffusion, pinocytosis, or possibly a receptor-mediated mechanism.

The question of differential uptake and retention of HPD by normal or malignant cells *in vitro* has been investigated by

a number of authors with conflicting results. An early report by Mossman et al. [1974] claimed greater uptake of HPD into malignant human cervical cells compared to normal cells. Similarly, Andreoni reported increased photosensitivity by a transformed rat thyroid cell line compared to normal rat thyroid cells [Andreoni et al., 1983]. However Chang and Dougherty [1978] reported malignant cells (HeLa, PC-1) accumulated HPD at the same rate as normal cell lines (CHO, L929). Moan et al. [1981] showed C3H cells of greater oncogenic potential accumulated slightly more HPD but had the same photosensitivity as normal cells. Bohmer and Morstyn [1985] also showed malignant and normal cells took up equivalent amounts of HPD. The conflict in the above results is not easily explained and may reflect differences in the cells and assay methods.

The stage of the cell cycle may affect the uptake of HPD and subsequent photosensitivity. There was no alteration in HPD uptake or photosensitivity throughout the cell cycle of synchronised Chinese hamster ovary (CHO) cells [Gomer and Smith, 1980]. However photosensitivity of NHIK 3025 cells after incubation with HP increased more than a hundred fold from early G1 to a maximum in mid S [Christensen et al., 1979]. There was increased uptake of HP in S phase [Christensen and Moan, 1980]. It was also suggested that the capacity for repair of sublethal damage may be greater in early G1 [Christensen and Moan, 1979]. Variations in the effect of cell cycle on uptake of porphyrin may reflect differences in the cell lines or the porphyrins.

The kinetics of the uptake and loss of HPD by cells

in vitro has been studied extensively. When cells are incubated in HPD, there is rapid initial uptake of HPD over the first 30min to 2h and slower uptake over the next 24h [Berns et al., 1983; Henderson et al., 1983; Hilf et al., 1983]. The HPD initially taken up by the cells is loosely bound and can be easily washed out of the cells with serum-rich medium [Christensen et al., 1983]. As the incubation time is extended, the proportion of tightly bound HPD in the cells increases [Henderson et al., 1983; Bellnier and Lin, 1983; Moan et al., 1984]. The tightly bound fraction appears to contribute far more to the photosensitivity of cells. Bellnier and Lin [1983] incubated cells with HPD under varying conditions which all resulted in the same intracellular concentration of HPD. Cells containing tightly bound HPD were more photosensitive than those containing loosely bound HPD. Similar experiments by Henderson et al. [1983] and Christensen et al. [1983] also showed that tightly bound HPD was a more efficient photosensitizer.

The chemical composition of the tightly bound porphyrin fraction and the sites of intracellular localization have been examined. The most hydrophobic porphyrins in HPD were taken up and retained selectively by cells [Moan and Sommer, 1984; Kessel, 1984a]. Hydroxyethyl vinyl deuteroporphyrin (HVD) and protoporphyrin (PP) were readily taken up by cells but easily washed out. The tightly bound porphyrins extracted from the cells were shown to be HP [Kessel and Chou, 1983]. Since HP is too hydrophilic to cross lipid rich membranes, intracellular conversion of the porphyrins, possibly by a gradual breakdown of hydrophobic aggregates, is likely.

Efficiency of photoinactivation of cells increased with

increasing fluorescence quantum yield. Since highly aggregated porphyrins have a low fluorescence quantum yield [Moan and Sommer, 1984], this suggests that the intracellular photoactive porphyrins are not highly aggregated. An increase in intensity of intracellular fluorescence was observed with increasing incubation time, suggesting that the porphyrins gradually disaggregate within the cells [Bellnier and Lin, 1983].

The location of intracellular porphyrin is dependent on the duration of incubation with HPD. A short incubation resulted in membrane damage after irradiation but a longer incubation resulted in DNA damage without damage to cell membranes [Christensen et al., 1983]. A long incubation may allow HPD to migrate to more sensitive internal sites within the cell. After incubating cells in HPD for 20h, fluorescence of intracellular porphyrin was localized in the perinuclear region, an area of high mitochondrial and lysosomal density [Berns et al., 1983; 1984a]. Sites of intracellular damage will be considered below.

(ii). Influence of serum on uptake of HPD

There is a strong affinity of binding between porphyrins and serum proteins, particularly albumin and haemopexin [Muller-Eberhard, 1970; Morgan and Muller-Eberhard, 1972; Muller-Eberhard and Morgan, 1975; Lamola et al., 1981]. Therefore, transport of HPD in the body must be largely mediated by serum proteins. At therapeutic concentrations, 85% of HPD is bound to human serum albumin [Grossweiner and Goyal, 1984]. A recent report has also suggested that lipoproteins may be carriers of porphyrins in the serum [Kessel et al., 1986].

Uptake of HPD by cells in vitro was reduced by 75% in the

presence of 10% serum, with a corresponding decrease in the efficiency of photoinactivation [Moan et al., 1979; Moan and Christensen, 1981]. Uptake of ^{3}H-HPD was reduced by serum, with 1% serum decreasing uptake of HPD by 50% [Gomer and Smith, 1980]. Serum inhibited the photodynamic killing of retinoblastoma cells but heat inactivation of serum had no additional effect, indicating that complement did not play a role in the inhibitory effect of serum [Sery, 1979]. Binding of HPD to cells may therefore be inhibited by competitive binding to serum proteins.

(iii). Uptake and retention of HPD in tumours

The use of fluorescence of HPD for diagnosis of tumours has been considered in section (b). Early studies of fluorescent detection of porphyrins showed tumours accumulated greater levels of porphyrin than normal tissues [Figge et al., 1956]. Carpenter et al. [1977] demonstrated that fluorescence was more intense at the periphery of tumours where the tumour invaded the surrounding normal tissue and blood vessels. Normal muscle did not fluoresce. Later studies, using radioactively labelled HPD, showed uptake in normal tissue as well as tumours. Higher levels of ^{3}H- and ^{14}C-HPD were detected in liver, kidney and spleen than in SMT-F mouse mammary carcinoma. There was less ^{3}H- or ^{14}C-HPD in skin, heart, muscle and brain than in tumour [Gomer and Dougherty, 1979]. These results were confirmed by Evensen et al. [1984a], who examined ^{3}H-HPD uptake in mice with Lewis lung carcinoma. There was greater uptake of ^{3}H-HPD in the eyes of mice with human retinoblastoma transplants than in normal eyes [Gomer et al., 1982]. Gomer and Dougherty [1979] claimed there was no exchange of the radioactive label on HPD, as ^{14}C-HPD and ^{3}H-HPD gave equal

tissue levels. However it is possible that the radioactivity detected in tissues may be a breakdown product of HPD. Gomer and Little [1984] demonstrated breakdown of ^{3}H-HPD into radioactive fragments, so results should be assessed with caution. Fluorescence uptake studies may also be criticized, as tissue, particularly if highly pigmented, may cause quenching of the fluorescence and thus not give an accurate indication of HPD levels.

Localization of HPD within tumours has been investigated both by fluorescence and by autoradiography with ^{3}H-HPD. The technical limitations mentioned above also apply here. ^{3}H-HPD autoradiography of SMT-F mouse tumour showed that the highest grain densities were over the pseudocapsule, necrotic areas and the vascular stroma of the tumour rather than in the tumour cells. The macrophages of the vascular stroma retained ^{3}H-HPD for the longest time [Bugelski et al., 1981].

The reasons for the relative specificity of uptake and retention of HPD in tumours are still unclear. Malignant cells do not appear to accumulate increased levels of HPD in vitro when compared to normal cells. Cozzani et al., [1981] showed clearance of porphyrins from malignant cells was slower than from normal cells. The altered vascularity of tumours (Chapter 9) may be important in determining specificity of uptake of HPD. The uptake and retention of HPD in phagocytic cells of the reticuloendothelial system suggests that phagocytosis may be a major mechanism of uptake. A constant ratio of extracellular to intracellular porphyrin in normal liver and ascites hepatoma, suggesting passive diffusion, has also been demonstrated [Cozzani et al., 1981].

The interstitial pH of tumours may also be an important factor in determining the specificity of uptake of HPD. The interstitial fluid of tumours has a low pH [Gullino et al., 1965]. Moan et al. [1980a] showed that cellular uptake of HP increased with decreasing extracellular pH, suggesting a lower pH may increase uptake of HPD into tumours.

(iv). Mechanisms of clearance of HPD

Porphyrins appear to be cleared from the body by metabolism in the liver and excretion through the biliary tract, with only a very small amount of porphyrin being metabolised by the tumour cells or excreted in the urine [Jori et al., 1979]. Carpenter et al. [1977] also observed intense fluorescence in the biliary tract.

(e). Mechanisms of cellular damage by HPD and light

(i). Oxygen dependency of the phototoxic process

Photodynamic inactivation of cells by porphyrins occurs only in the presence of oxygen. Studies by a number of workers confirm this.

Deoxygenation of culture medium by bubbling with nitrogen inhibited inactivation of Saccharomyces cerevisae by HP and light [Kvello Stenstrom et al., 1980]. Photodynamic inactivation of Herpes Simplex virus by HPD and light was also inhibited in deoxygenated medium [Lewin et al., 1980]. However, phosphatidylcholine lysosomes sensitized with HP were lysed when irradiated under nitrogen flushing conditions [Grossweiner et al., 1982]. Oxygen tension was not measured, so it is possible lysis was mediated by trace amounts of oxygen.

The formation of an HP radical after irradiation was demonstrated by electron spin resonance (ESR) spin trapping. N_2

flushing inhibited production of the radical, indicating its production was dependent on the presence of oxygen [Moan et al., 1979].

The absolute requirement for oxygen for the photodynamic destruction of Raji cells sensitized with HPD was shown by Lee See et al. [1984]. Graded amounts of sodium dithionite were added to phosphate buffered saline (PBS) to give precise measured oxygen tensions. At a pO_2 of 0, there was no photodynamic inactivation of Raji cells. With increasing oxygen tension, there was a proportional increase in photocytotoxicity. Hypoxic V79 cells were also extremely resistant to photodynamic damage mediated by HPD [Mitchell et al., 1985]. Moan and Sommer [1985] showed efficiency of photoinactivation of HPD-sensitized NHIK 3025 cells decreased with decreasing oxygen concentration and photoinactivation did not occur in N_2.

The requirement for oxygen for photodynamic damage has also been demonstrated in vivo. Clamping of mouse legs prior to PDT completely abolished photodynamic damage to the skin. This was attributed to reduced oxygen tension [Gomer and Razum, 1984].

The dependence on oxygen for photodynamic damage implies that hypoxic areas of tumours may be inadequately treated. However complete tumour regressions after PDT have been extensively reported (see (c).). Tumour necrosis can not depend entirely on direct photodynamic destruction of tumour cells but alternative mechanisms of tumour destruction such as vascular damage must also be important.

(ii). Role of singlet oxygen and other toxic oxygen species

The requirement for oxygen in photodynamic damage mediated

by HPD been discussed above. The nature of the active oxygen species and the mechanisms of action have been intensively studied.

Singlet oxygen ($^1O_2$) was first implicated in the photodynamic activity of HPD by Weishaupt et al. [1976]. 1,3-diphenylisobenzofuran completely inhibited the photodynamic inactivation of TA-3 mouse mammary carcinoma. Quenching by furans of the photodynamic activity of porphyrins has also been reported by other authors [Bodaness and Chan, 1977; Ito, 1978; Dixit et al., 1983; Das et al., 1985], supporting the hypothesis that $^1O_2$ plays a major role in the photoactivity of porphyrins. However $^1O_2$ is not the only species which causes the degradation of furans. Almost any oxidant (eg. halogens, peracids or air) will convert furans to diketones [Krinsky, 1979].

Deuterium enhancement is commonly used to detect $^1O_2$. An increase in the lifetime of $^1O_2$ from 2 to 20 microseconds results in a ten fold increase in the efficiency of photooxidation in D_2O compared to water [Merkel and Kearns, 1972]. Enhancement of HPD- or HP-mediated phototoxicity in deuterated buffers has been reported. Photoinactivation of NHIK 3025 cells by HP and light was enhanced in deuterated buffer [Moan et al., 1979; Moan and Boye, 1981]. Deuterated medium potentiated the inactivation of yeast cells by HP and light [Kvello Stenstrom et al., 1980; Ito, 1981]. There was a greater quantum yield of $^1O_2$ in deuterated buffers after HPD solutions were irradiated [Moan and Sommer, 1981]. Photooxidation of NADPH by HP was also enhanced in D_2O [Bodaness and Chan, 1977]. ^{51}Cr release after irradiation of HPD-sensitized Raji cells was

enhanced in D_2O buffer [Lee See, 1985].

Photooxidation of tryptophan has been used to measure $^1O_2$ [Moan and Sommer, 1981; Moan, 1984]. Grossweiner [1984] claimed tryptophan oxidation in human serum albumin/HP complexes was mediated by $^1O_2$. Irradiation of HPD solutions resulted in tryptophan degradation [Lee See, 1985]. However tryptophan may be degraded through a type 1 reaction (electron transfer from the triplet state of HP) without any involvement of $^1O_2$ [Rossi et al., 1981].

Azide, histidine, methionine, beta-carotene and ascorbic acid have all been claimed to be specific quenchers of $^1O_2$. Yeast cells sensitized with HP were protected from photodynamic damage by sodium azide [Kvello Stenstrom et al., 1980; Ito, 1981]. The photooxidation of NADPH by HP was quenched by histidine and methionine [Bodaness and Chan, 1977]. Destruction of cytochrome P-450 by HPD and light was inhibited by histidine, beta-carotene and ascorbic acid [Dixit et al., 1983; Das et al., 1985]. ^{51}Cr release after irradiation of HPD-sensitized Raji cells was inhibited by both azide and histidine [Lee See, 1985].

Vitamin E (alpha-tocopherol) has been claimed to be a $^1O_2$ quencher although it also traps other free radicals [Forman and Fisher, 1981]. Vitamin E protected lysosomes sensitized with HP from photodynamic damage [Torinuki et al., 1980a; 1980b] and also protected PP-sensitized fibroblasts against photolysis [Wakulchik et al., 1980].

The hydroxyl radical ($^{\bullet}OH$) has also been implicated in the photodynamic activity of HPD. Benzoate, mannitol, ethanol thiourea, formate, dimethyl sulphoxide and albumin have all been claimed to be quenchers of $^{\bullet}OH$ [Bors et al., 1982].

However reactions may also occur between these quenchers and other radicals. Photodynamic destruction of HPD-sensitized cytochrome P-450 was inhibited by benzoate, mannitol and ethanol [Dixit et al., 1983; Das et al., 1985] but photooxidation of NADPH by HP was not inhibited by benzoate or mannitol [Bodaness and Chan, 1977]. This may reflect differences in the experimental systems and the photosensitizing porphyrin. Dimethyl sulphoxide, ethanol and mannitol inhibited ^{51}Cr release after HPD-sensitized Raji cells were irradiated [Lee See, 1985].

Degradation of hyaluronic acid has been used to detect $^{\bullet}OH$ in non-cellular systems [Betts and Cleland, 1982]. Lee See [1985] showed hyaluronic acid degradation occurred after irradiating HPD, implying that $^{\bullet}OH$ was produced.

Hydroxyl radicals may be detected using appropriate spin trapping compounds in electron spin resonance (ESR). Specific $^{\bullet}OH$ adduct signals were detected after irradiating solutions of HPD or Photofrin II [Buettner, 1985; Buettner and Need, 1985]. Further evidence for the production of $^{\bullet}OH$ by HPD was also provided by demonstrating the formation of thymine glycols in the DNA of PDT-treated cells [Hariharan et al., 1980].

The role in PDT of other active oxygen species, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), is unclear. Spin adducts from O_2^- were detected by ESR after irradiation of PP [Buettner and Oberley, 1979] and HP [Cox et al., 1982]. Peterson et al. [1981] suggested that irradiation of HPD generated O_2^-, since nitro blue tetrazolium (NBT) was reduced in the photodynamic reaction and the reduction was inhibited by superoxide dismutase (SOD). In contrast, Gibson et al. [1984]

showed ferricytochrome C and NBT were not reduced after irradiation of HPD. SOD did not inhibit photodestruction of either HPD-sensitized cytochrome P-450 [Dixit et al., 1983; Das et al., 1985] or NADPH [Bodaness and Chan, 1977]. Lee See did not detect any inhibition by SOD of ^{51}Cr release from HPD-sensitized Raji cells after irradiation [Lee See, 1985]. Therefore it is unlikely that O_2^- causes photodynamic damage to cells, although O_2^- may be produced by irradiating HPD in non-cellular systems.

Production of H_2O_2 may be deduced from the protective effect of catalase on photodynamic reactions. This has not been observed with porphyrins. HPD-sensitized photoinactivation of cytochrome P-450 was not inhibited by catalase [Dixit et al., 1983; Das et al., 1985]. Lee See [1985] did not observe inhibition by catalase of ^{51}Cr release from Raji cells after PDT. In contrast, a recent report by Buettner and Need [1985] suggested that H_2O_2 was produced after irradiation of Photofrin II. Detection of the $^{\bullet}OH$ spin adduct by ESR was inhibited by catalase, implicating H_2O_2 in the production of $^{\bullet}OH$. However this does not mean that H_2O_2 plays a primary role in photodynamic damage to cells, but it may be a precursor of a cytotoxic species, possibly $^{\bullet}OH$.

In conclusion, there is considerable evidence for the production of $^1O_2$ and $^{\bullet}OH$ when HPD is irradiated and these toxic species cause photodynamic damage to cells. When considering the role of toxic oxygen species in the photodynamic inactivation of cells, the lack of specificity of some of the assays for a single reactive species must be considered. The roles of O_2^- and H_2O_2 are less clear but they are probably not the main species causing damage to cells

during PDT.

(iii). Membrane protein damage

Photoactivation of intracellular porphyrin causes membrane damage resulting in cell lysis. Membrane proteins are important sites of photodynamic damage and the nature of this damage will be considered below.

Photodynamic damage to cell membranes has been demonstrated in morphological studies. Electron microscopy of NHIK 3025 cells after PDT showed membrane vesiculation, cell swelling and reduction in the number and size of microvilli [Moan et al., 1982a]. Photodynamic damage to HP-sensitized lymphoma cells resulted in the formation of vacuoles and uropod-like structures, followed by disruption of the plasma membrane [Coppola et al., 1980].

Membrane damage resulting from the photoinactivation of cells can be shown by the release of ^{51}Cr [Ranadive et al., 1979; Bellnier and Dougherty, 1982]. ^{51}Cr release corresponded with cell death as measured by colony formation. This will be discussed in more detail in Chapter 2.

Photodynamic damage to cell membranes has been studied using erythrocyte ghosts as model systems. After treating ghosts with PP and light, formation of large aggregates of membrane proteins was demonstrated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). The aggregates were stable in SDS-8M urea, indicating the formation of covalent bonds. A loss of histidine, cysteine, tyrosine and tryptophan from membranes suggested that photooxidation of these amino acids resulted in cross-linking of membrane proteins [Dubbelman et al., 1978; Girotti, 1979]. The major

cross-links were between histidine-sulphydryl groups and histidine-arginine [Vereweij and Van Steveninck, 1982], which suggests $^1O_2$ is involved in the formation of cross-links, since histidine is a quencher of $^1O_2$. Carbohydrate residues were not susceptible to photodamage and peroxidation of unsaturated fatty acids was not involved in the cross-linking of proteins [DeGoeij et al., 1976]. A greater number of protein aggregates were formed if the cells were irradiated at 0^o, suggesting membrane fluidity was a factor in photodynamic damage [Dubbelman et al., 1980a]. Cross-linking was initiated by irradiation of photosensitized cells but continued after irradiation. Cross-linking may therefore be a secondary process between photooxidised amino acids and other reactive groups, rather than the primary event causing photodynamic damage to cells.

Photodynamic damage to cells modifies the function of membrane-bound enzymes and membrane proteins involved in transport processes. Uptake of nucleosides and amino acids was inhibited after treating L1210 cells with HP or PP and light [Kessel, 1977a; 1977b]. Cross-linking of membrane proteins correlated with a decrease in cycloleucine transport after PDT [Kohn and Kessel, 1979]. Photooxidation of membrane proteins inhibited active transport of glucose, L-leucine, sulphate and glycerol into erythrocytes [Dubbelman et al., 1980b]. Active transport of Na^+ and K^+ was inhibited by photodynamic damage to Na^+K^+-dependent ATPase [Schothorst et al., 1971]. The active transport systems for aminoisobutyric acid and Rb^+ in L292 fibroblasts were also sensitive to photodynamic inactivation by HPD [Dubbelman and Van Steveninck, 1984]. However these

transport systems were damaged by lower light doses than those required for the formation of protein cross-links, indicating that cross-links represent secondary damage and need not occur for the deterioration of membrane function.

(iv). Membrane lipid damage

The production of toxic oxygen species after photoinactivation of HPD results in the photooxidation of lipids in the cell membrane. As discussed above, lipid peroxidation does not lead to the formation of protein cross-links but membrane lipid damage will lead to alterations in membrane permeability and function which may kill the cell.

HPD-sensitized phosphatidyl choline liposomes undergo lipid peroxidation and lysis after irradiation [Goyal et al., 1983; Grossweiner, 1984; Reyftman et al., 1985]. Malondialdehyde formation, which results from lipid peroxidation, was measured after irradiation of PP-sensitized erythrocyte ghosts [Girotti, 1979; Girotti et al., 1985]. Lipid peroxidation has also been detected after irradiating HP-sensitized erythrocytes [Sorata et al., 1984]. This was suppressed by sodium azide, indicating that lipid peroxidation was mediated by $^1O_2$. Cholesterol in HP-sensitized liposomes was also photooxidised, confirming $^1O_2$ was involved in the photodynamic process [Suwa et al., 1977].

(v). DNA damage

Although the cell membrane is a major site of photodynamic damage, there is evidence that the cellular DNA is also damaged by PDT. This is very important, since any alterations to the DNA may lead to mutations and possibly the development of cancer.

Porphyrins bind and intercalate into isolated DNA [Fiel et

al., 1979]. The photodynamic modification of DNA by HP resulted in selective photooxidation of guanine only, with cross-links formed between guanine and tryptophan or histidine [Gutter et al. 1977; Dubbelman et al., 1982]. Irradiation of HPD-sensitized isolated DNA caused both single and double strand breaks [Fiel et al., 1981; Boye and Moan, 1980].

Photodynamic damage to the DNA of intact cells has been reported by a number of authors. Blazek and Hariharan [1984] detected DNA strand breaks and covalent DNA-protein cross-links after irradiation of HPD-sensitized cells. Repairable DNA strand breaks were caused by irradiation of HPD-sensitized cells but at a lower frequency than that occurring after X-ray treatment [Gomer, 1980; Moan et al., 1980b].

Sister chromatid exchange has been demonstrated after irradiation of photosensitized cells [Moan et al., 1980b; Christensen et al., 1983]. In contrast, Waksvik et al., [1980] did not show an increased frequency of sister chromatid exchanges after irradiating HP-sensitized cells. Chromosomal aberrations localized around the telomeric and centromeric regions were also reported [Evensen and Moan, 1982].

The occurrence of DNA and chromosome damage after PDT suggests that PDT may cause mutations or the development of cancer. Gruener and Lockwood [1979] considered whether $^1O_2$ may act as a primary mutagen or carcinogen. The number of oubain-resistant mutants produced in CHO cells by treatment with Rose bengal and light was enhanced by D_2O and reduced by $^1O_2$ quenchers, suggesting that $^1O_2$ causes mutations. A recent study by Gomer et al. [1984b] examined the mutagenic potential of HPD-PDT. The rate of mutation causing resistance to 6-

thioguanine in CHO cells was examined. In contrast to U.V. or X irradiation, PDT did not increase the rate of mutation. The low rate of DNA strand breaks after PDT compared to ionising radiation, also suggests cellular mutation may not be common with PDT.

(vi). Mitochondrial damage

When HPD is taken up into isolated cells, it is associated with the mitochondria [Berns et al., 1982]. Fluorescence microscopy demonstrated a perinuclear pattern of HPD uptake, which correlated with the distribution of mitochondria and lysosomes in the cells. The large mitochondria of myocardial cells were also fluorescent.

Treatment of isolated mitochondria with PP and light resulted in uncoupling and inhibition of oxidative phosphorylation, energy dissipation, and inhibition of respiration followed by swelling and disruption of the mitochondria [Sandberg and Romslo, 1980]. The Ca^{2+} pump was inhibited after treating isolated mitochondria with HP or HPD and light [Salet and Moreno, 1981; Salet et al., 1984]. Photodynamic damage to PP-sensitized mitochondria was enhanced by D_2O and inhibited by azide, suggesting $^1O_2$ was the toxic agent [Sandberg and Romslo, 1981].

Photodynamic inactivation of mitochondrial enzymes has also been demonstrated. Proton-translating ATPase [Perlin et al., 1985], cytochrome C oxidase [Gibson and Hilf, 1983], and succinate dehydrogenase [Hilf et al., 1984a; 1984b] were all inhibited by HPD and light. Enzymes associated with the mitochondrial membrane were more susceptible to photodynamic damage than those located in the mitochondrial matrix or in the cytosol [Hilf et al., 1984a], suggesting that mitochondrial

membranes may be a primary site of photodynamic damage.

Morphologic studies have demonstrated that mitochondrial damage is detected soon after irradiation of HPD-sensitized cells. Mouse lymphoma cells treated with HPD and light were examined by electron microscopy [Coppola et al., 1980]. The earliest detectable damage was mitochondrial contraction followed by swelling and ruptured cristae. Similarly, shrinkage of the mitochondria was the first sign of damage after irradiation of HPD-sensitized NHIK 3025 cells [Moan et al., 1982a]. Mitochondrial damage in human adenocarcinoma cells after PDT was demonstrated by electron microscopy [Tatsuta et al., 1984]. Moreno and Salet [1985] used laser microirradiation on single myocardial cells sensitized with HPD. At low light doses, irradiation of the mitochondria caused cell death but irradiation of the nucleus or cytoplasm had no effect. Mitochondrial damage was also an early event after irradiation of a rat transitional cell bladder tumour in the intact animal [Klaunig et al., 1985].

Mitochondria preferentially accumulate HPD and show the earliest detectable photodynamic damage. Inactivation of respiratory chain enzymes associated with the mitochondrial membrane results in loss of mitochondrial function and rupture, hence cellular inactivation.

(vii). Lysosomal damage

Lysosomes accumulate HPD, shown by fluorescence in the perinuclear region of cells which is an area of high lysosome concentration [Berns et al. 1982].

Lysosomal damage mediated by porphyrins and light was demonstrated by Allison et al. [1966] and Slater and Riley

[1966]. Lysosomal enzymes, acid phosphatase and beta-glucuronidase, were released after irradiation of HP-sensitized isolated lysosomes. Malondialdehyde formation, indicating lipid peroxidation, was also detected [Torinuki et al., 1980a; 1980b]. The lysosomal enzyme, N-acetyl-beta-D-glucosaminidase was released from HP-sensitized human fibroblasts after irradiating with low light fluxes that resulted in minimal morphological damage [Santus et al., 1983]. Lysosomal damage may therefore be an early event in photodynamic damage to cells.

However other studies have suggested lysosomal damage may not be important for photocytotoxicity. Lysosomal enzymes were released after irradiation of HPD-sensitized NHIK 3025 cells but leupeptin, a lysosomal enzyme inhibitor, did not protect the cells from photodynamic damage [Christensen et al., 1982]. PP-sensitized fibroblasts [Wakulchik et al., 1980] or isolated lysosomes [Sandberg, 1981] did not release lysosomal enzymes after irradiation. The apparent contradictions observed in the susceptibility of lysosomes to photodynamic damage may possibly be due to the different porphyrins. Lysosomes sensitized with HP but not with PP were damaged by irradiation. Differences in hydrophobicity of the two porphyrins may modify the uptake into lysosomes and hence photosensitivity.

Therefore, while lysosomes are damaged in photodynamic reactions, it is not clear if this is important for the inactivation of cells.

(viii). Vascular damage

As discussed earlier, uptake and retention of HPD is greater in the vascular stroma of tumours than the tumour cells [Bugelski et al., 1981]. HP binds to fibrinogen and prolongs

the clotting time after irradiation [Zieve and Solomon, 1966; Musser et al., 1979]. This could result in haemorrhage in tumours. It is therefore likely that the tumour vasculature will play an important role in tumour destruction.

There have been a number of reports of vascular damage in tumours after PDT. An increase in vascular permeability was observed after irradiating normal rat ears sensitized with porphyrin C [Barker et al., 1970]. Irradiation of HP-sensitized Yoshida ascites hepatoma in rats resulted in massive coagulation necrosis 24h after irradiation [Tomio et al., 1983]. Damage to the tumour vasculature during PDT has been observed directly by Star et al. [1984] who grew rat mammary carcinoma in "sandwich" observation chambers. As HPD-sensitized tumours were irradiated, blanching of the small blood vessels was observed, followed by a complete stop in tumour microcirculation. The blood flow in the tumour vasculature was more sensitive to PDT than in normal blood vessels.

Tumour blood flow (measured with radiolabelled microspheres) was significantly reduced after irradiation of HPD-sensitized AY-27 rat urothelial tumours [Selman et al., 1984; 1985]. The reduction in blood flow correlated with tumour regression.

Studies in vitro have also provided evidence that damage to vasculature rather than to tumour cells causes photodynamic damage to tumours. Musser and Data-Gupta [1984] demonstrated tumour necrosis after irradiating HPD-sensitized L1210 solid tumours in mice. However, when they injected HPD into a tumour bearing mouse and isolated the tumour cells, these cells were not killed by irradiation in vitro and contained very low

levels of intracellular HPD. This implies that tumour necrosis *in vivo* could not have resulted from direct tumour cell destruction. The same conclusion was reached by Henderson et al. [1984, 1985]. HPD was administered to mice with EMT-6 tumours and the tumours irradiated. Tumours were removed at varying times after irradiation and the tumour cells assayed for viability by colony formation. There was no reduction in viability of tumour cells isolated directly after irradiation but viability *in vitro* was reduced if the tumours were left in the mice. This reduction corresponded to the rate of reduction in viability if the blood vessels leading to the tumours were clamped. This suggests one of the main factors causing tumour destruction may be occlusion of the vascular system.

(ix). Effect of hyperthermia on efficacy of PDT

The light fluxes used in PDT vary considerably, but may be as high as 600mW with total energy delivered up to 360J [Dougherty et al., 1985]. These light intensities may be expected to heat the tumours and surrounding tissue. During irradiation, temperatures of up to 50^{o} at the point of insertion of a fibre delivering 200mW were measured. Tumour regressions without HPD sensitization were attributed to hyperthermia [Kinsey et al., 1983]. Berns et al. [1984] recorded similar temperature rises in tumours during irradiation.

Lipson and Baldes [1960b] first suggested that hyperthermia may act in combination with PDT. They showed an additive effect of PDT followed by heat on the efficacy of destruction of mouse tumours. Since this initial report, enhancement by hyperthermia of the response to PDT has been noted by a number of authors. Doses of heat that did not cause

tumour or tumour cell destruction alone, caused enhancement of PDT [Melloni et al., 1984; Waldow and Dougherty, 1984; Waldow et al., 1984; Christensen et al., 1984]. The sequence of PDT and hyperthermia is important in modifying the efficacy of PDT. Heat delivered immediately after irradiation is synergistic with PDT, but the synergistic effect is reduced by a greater interval between PDT and heat. Heating of the tumour before irradiation is only additive with PDT and not synergistic [Waldow et al., 1985]. Hyperthermia reduces tumour blood flow [Bicher et al., 1981]. This could result in increased vascular damage and more efficient tumour destruction.

Tumour destruction by PDT does not result from a pure photodynamic reaction, with heating of the tumours also being a factor. However, this is not necessarily a disadvantage and localized heating applied immediately after irradiation may improve efficacy of treatment.

(x). Cytotoxicity of HPD without light

Although the combination of HPD plus light is required for tumour necrosis *in vivo*, there have been a number of reports that HPD alone has cytotoxic effects on isolated cells.

The growth of rat kangaroo epithelial kidney cells was inhibited by HPD without irradiation [Berns et al., 1982]. HPD alone inhibited incorporation of ^{3}H-thymidine into L1210 cells *in vitro* [Franco et al., 1981]. Leukaemic cells were killed by PP without irradiation, but normal lymphocytes were resistant to non-phototoxic damage [Malik and Djaldetti, 1980a; 1980b]. PP alone supressed protein synthesis by human granulocytes, lymphocytes and platelets [Malik et al., 1979].

(f). Chemical composition of HPD

Haematoporphyrin derivative (HPD) was first described by Lipson [Lipson and Baldes, 1960a; Lipson et al., 1961a; 1961b] as a tumour localizer superior to HP. HPD is a complex mixture of porphyrins and since these initial studies, there has been considerable effort expended to determine the chemical composition of HPD and the nature of the porphyrin(s) responsible for the tumour localizing and phototoxic properties.

The chemical composition of HPD and the nature of the active species will be considered in Chapters 4 and 5.

(g). Cutaneous photosensitivity

The major side effect of PDT is severe cutaneous photosensitivity for 4 to 6 weeks after HPD infusion. This limits the application of PDT, particularly in minor cases, such as basal cell carcinoma, where effective PDT is possible but alternative forms of therapy may be preferred to avoid photosensitization of the patient. Methods to minimise photosensitivity should be intensively investigated.

(i). Use of quenchers to modify photodamage

Quenchers of $^1O_2$ or other toxic oxygen species may be useful in minimising cutaneous photosensitivity. Quenchers have been used to control cutaneous photosensitivity in patients with erythropoietic protoporphyria where elevated levels of PP are found in the skin. Beta-Carotene partially protected both HP-sensitized erythrocytes from photohaemolysis and mice sensitized with HP against photodynamic skin damage [Moshell and Bjornson, 1977]. Mice with chemically induced porphyria were partially protected by beta-carotene from skin damage

after irradiation [Mathews-Roth, 1984]. Beta-carotene is partially effective in reducing photosensitivity in patients with erythropoietic protoporphyria [Mathews-Roth, 1982]. There have not been any reports of the efficacy of beta-carotene in reducing cutaneous photosensitivity after administration of HPD. Other quenchers of reactive oxygen species (see (e).(i).) should also be examined for their efficacy in reducing cutaneous photosensitivity.

(ii). Methods to improve specificity of uptake of HPD

Cutaneous photosensitivity could also be minimised by improving the ratio of HPD uptake between tumour and skin or other normal tissues. This would allow a lower HPD dose to be administered to achieve the same concentration in tumours.

The therapeutic ratio could be improved by administering only the active fraction of HPD if this fraction had greater tumour specificity. A smaller total dose of porphyrin could also be administered. This approach to improving the efficacy of PDT will be considered in detail in Chapters 4 and 5.

New porphyrins with different tumour specificity or with more rapid clearance from the body to reduce the duration of photosensitivity, may also be useful. As an example, the photoactivity of porphyrin C, both *in vitro* and *in vivo*, will be considered in Chapters 4 and 5.

Coupling porphyrin to a tumour-specific antibody to target the delivery of porphyrin to the tumours may improve the therapeutic ratio. Mew et al., [1983] coupled HP to a monoclonal antibody directed against a mouse tumour. Antibody-specific uptake of the HP-antibody conjugate with conservation of the phototoxic properties of HP was demonstrated. This approach to improving the efficacy of treatment will be

considered in Chapter 6.

Liposomes may be potentially useful to improve the uptake of porphyrins into tumours. Irradiation of liposomes sensitized with HPD resulted in peroxidation of the liposomal membrane [Goyal et al., 1983]. HP incorporated into liposomes was taken up into HeLa cells more readily than free HP, resulting in greater photodamage to the cells [Cozzani et al., 1985]. Increased preferential uptake of liposome-bound HP into Yoshida hepatomas in rats was reported, with a tumour : liver ratio of 34:1 compared to 4:5 with free HP [Jori et al., 1983]. These results suggest liposomes may potentially increase preferential uptake of porphyrins into tumours. Liposomes are readily taken up by phagocytic cells but do not migrate across capillary walls and would not reach extravascular tumour tissues [Poste et al., 1982]. This may limit the usefulness of liposomes to deliver porphyrins to tumours unless the vascular system is the main site of damage for tumour destruction and photosensitization of the tumour cells themselves is not important.

CHAPTER 2

VALIDATION AND CHARACTERISTICS OF ^{51}Cr RELEASE AS AN ASSAY OF PHOTOCYTOTOXICITY IN VITRO

(a). Introduction

To examine the photodynamic activity of porphyrins *in vitro*, an assay was required that would provide a rapid and reliable measure of cellular damage by porphyrins and light. The sensitivity of the assay had to be adequate to detect differences in the photoactivity of the porphyrins. It was also hoped that some conclusions about the mechanism of the photocytotoxic process could be drawn from results of the ~~the~~ assay.

^{51}Cr release was examined as a measure of cytotoxicity. It has been used extensively to measure cell lysis, particularly in antibody-dependent cell mediated cytotoxicity assays [Sullivan et al., 1972; Wigzell, 1965], and reflects membrane damage [Brunner et al., 1968; Henney, 1973]. ^{51}Cr binds to intracellular proteins [Bunting et al., 1963; Ronai, 1969] and its release parallels membrane damage demonstrated by trypan blue uptake [Sullivan et al., 1972] and by histamine release from mast cells [Ranadive et al., 1979].

Other parameters of cytotoxicity were also examined to assess the validity of ^{51}Cr release as a measure of phototoxicity. Inhibition of ^{3}H-thymidine uptake and of colony formation on semisolid agar after irradiation of HPD-sensitized Raji cells were investigated to determine if the capacity of a cell to divide could be damage by PDT without causing ^{51}Cr release.

(b). ^{51}Cr release assay

Methods

Raji cells, a B cell lymphoblastoid cell line derived from Burkitt's lymphoma [Minowada et al., 1978], were obtained from Dr. H. Zola, Department of Clinical Immunology, Flinders Medical Centre. Cells were grown in flat plastic flasks (Lux) at 37° in 5%CO_2 in RPMI 1640 (Flow Laboratories) buffered to pH7.4 with 25mM n-2-hydroxyethylpiperazine-N^1-2-ethane sulphonic acid (HEPES, Sigma) and 2.3mM $NaHCO_3$ and supplemented with 0.16ug/ml gentamicin and 10% foetal calf serum (FCS, Flow Laboratories). Cells were harvested when approaching stationary phase (10^6/ml) and subcultured by diluting in fresh medium to approximately 10^5/ml.

HPD was prepared in the Pharmacy of the Queen Elizabeth Hospital by a previously published procedure [Forbes et al., 1980]. One part of haematoporphyrin hydrochloride (Roussel) was dissolved in 20 parts of 19:1 v/v glacial acetic acid:sulphuric acid and allowed to stand overnight at room temperature. The solution was adjusted to pH6.0 by adding 3% sodium acetate and the precipitate collected by filtration through a Whatman No.1 filter paper. Injectable solution was prepared by dissolving one part HPD solid with 50 parts 0.1N NaOH and stirring for 1h at room temperature. The pH was adjusted to 7.4 with 0.1N HCl, and the solution diluted to 5mg/ml with 0.9% sodium chloride solution.

Raji cells (10^7/ml) were suspended in RPMI 1640/10%FCS and 50uCi/ml ^{51}Cr-sodium chromate (Amersham Australia), incubated for 1h at 37° and washed three times in RPMI 1640 [Ford and Hunt, 1973]. ^{51}Cr-labelled Raji cells (10^7/ml in RPMI 1640) were incubated with HPD for 1h at 37°, washed once in RPMI 1640

to remove unincorporated HPD and resuspended at $0.5X10^6$/ml in Dulbecco's phosphate buffered saline (PBS, Commonwealth Serum Laboratories). One ml aliquots in polycarbonate tubes were irradiated for 2-20min on a horizontal platform revolving around a 250W quartz iodide lamp with a red perspex filter. A fan blowing on the apparatus maintained the temperature at 25^o. The flux density at the point of irradiation was 8mW/sq cm between 600 and 650nm [Wilksch, 1982; unpublished data]. The tubes were centrifuged at 400g for 10min, 0.5ml of the supernatant (S) removed and the cell button plus 0.5ml supernatant (R) were counted in a Roche Gammamatic gamma counter. Percentage ^{51}Cr release was calculated from the formula: 2S/R+S x 100 [Timonen and Saksela, 1977]. Unless otherwise specified, ^{51}Cr release was determined immediately after irradiation.

Maximum ^{51}Cr release was determined after adding 100ul of ^{51}Cr-labelled cells ($5X10^6$/ml) to 900ul of 3% acetic acid. In a number of experiments, the maximum ^{51}Cr release varied between 75 and 85%. Unless otherwise specified, the percentage specific ^{51}Cr release was calculated by subtracting the background percentage ^{51}Cr, from cells incubated in HPD but not exposed to light, from all experimental values. Each point on the graphs represents the mean $\pm$ standard deviation (S.D.) of triplicates. Control cells irradiated with red light but not exposed to HPD were also tested in all experiments. Light alone did not increase ^{51}Cr release above background.

Uptake of HPD into Raji cells was assessed by examining fluorescence of cell suspensions under a Zeiss Fluorescence microscope with excitation wavelength 420-490nm.

Results

The relationship between light dose and ^{51}Cr release after irradiation of HPD-sensitized Raji cells is illustrated in Fig. 2.1. When low doses of HPD were used, several minutes irradiation was required before ^{51}Cr release began, then linear ^{51}Cr release until the maximum (75-80%) was reached.

The effect of varying the concentration of HPD with constant light dose is shown in Fig. 2.2. ^{51}Cr-labelled cells were incubated in 0-300ug/ml HPD, then irradiated for 2min. There was a threshold dose of HPD (approximately 50ug/ml), below which 2min irradiation was not phototoxic, then linear ^{51}Cr release occurred as HPD concentration was increased until a plateau of ^{51}Cr release was reached.

The relationship between ^{51}Cr release and the duration of exposure of the cells to HPD was tested. ^{51}Cr-labelled Raji cells were incubated with HPD (25ug/ml) at 37^{o} for 0-4 hours, then centrifuged and resuspended in PBS. The cells were irradiated for 0-20min and the percentage ^{51}Cr release determined as above. With increasing incubation time, there was a rapid increase in ^{51}Cr release at a given light dose (Fig. 2.3). A plateau was reached after approximately 1h and only a slight increase in ^{51}Cr release occurred after longer incubations. Thus cells were routinely incubated in HPD for 1h unless otherwise specified.

^{51}Cr-labelled cells were suspended in HPD (25ug/ml) and immediately centrifuged without incubation. Despite the very short contact time between HPD and the cells, higher light doses resulted in some ^{51}Cr release (Fig. 2.4). Rapid initial binding or uptake of HPD into the cells must cause rapid photosensitization. Red fluorescence was detected around the

Fig. 2.1

Relationship between light dose and percentage ^{51}Cr release. ^{51}Cr-labelled Raji cells were incubated for 1h with HPD, resuspended in PBS and irradiated for 1-20 min.

● 10ug/ml HPD

○ 25ug/ml HPD

■ 50ug/ml HPD

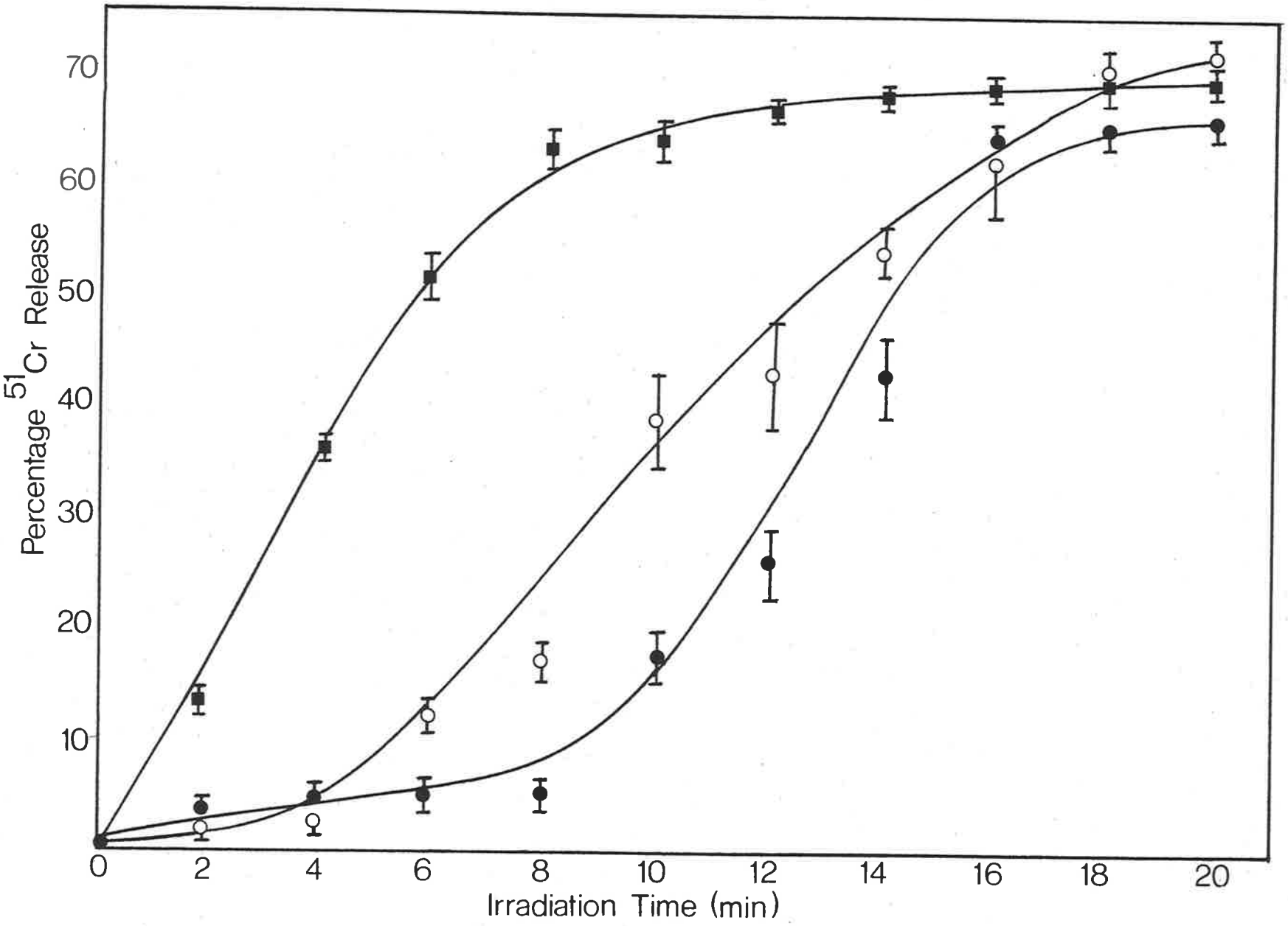

Percentage ^{51}Cr Release
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
Irradiation Time (min)

Fig. 2.2

Relationship between Percentage ^{51}Cr release and dose of HPD

^{51}Cr-labelled Raji cells were incubated for 1h with graded doses of HPD, resuspended in PBS and irradiated.

● 2min irradiation

O No irradiation

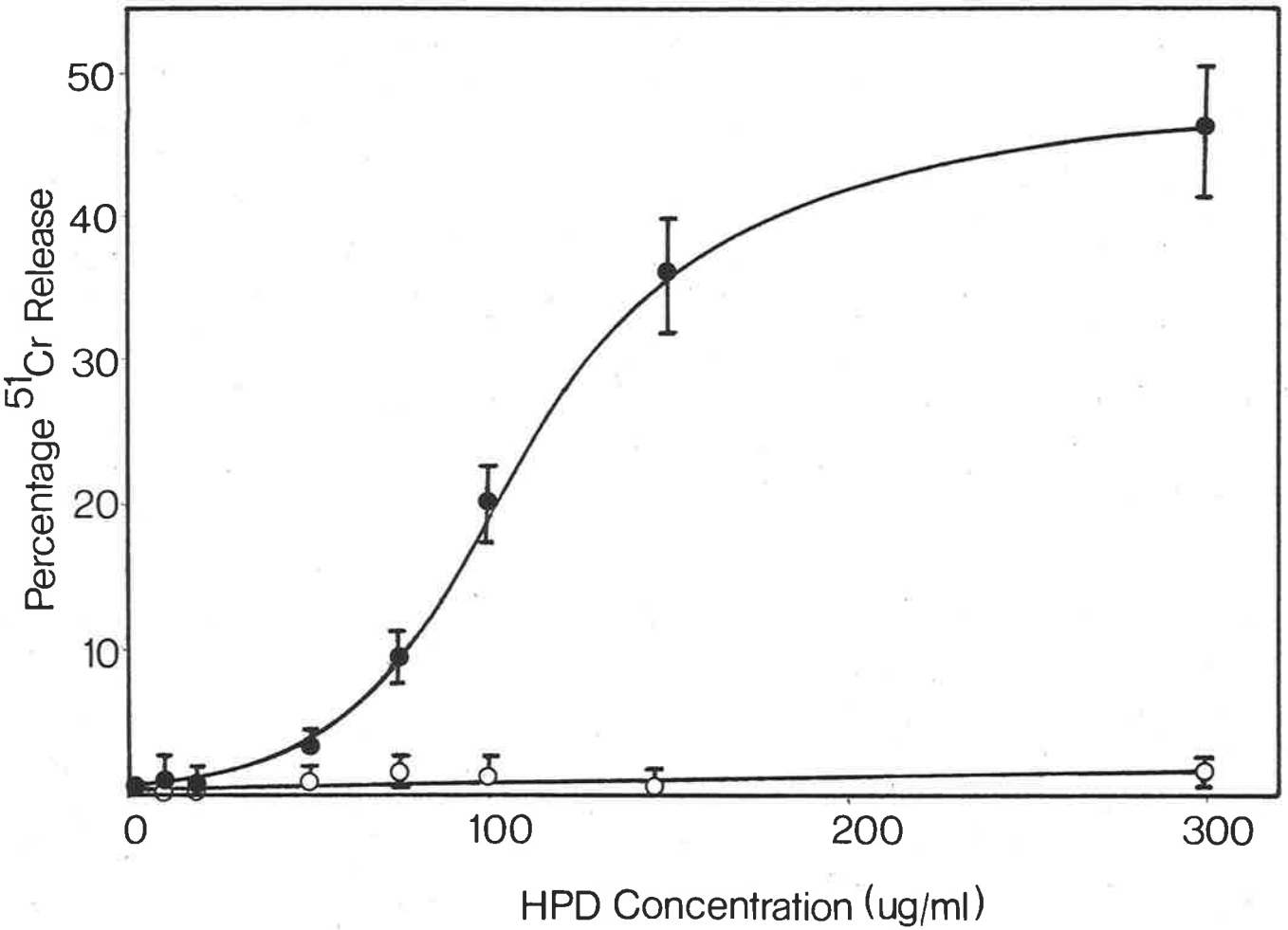

50
40
30
20
10
Percentage ^{51}Cr Release
0
100
200
300
HPD Concentration (ug/ml)

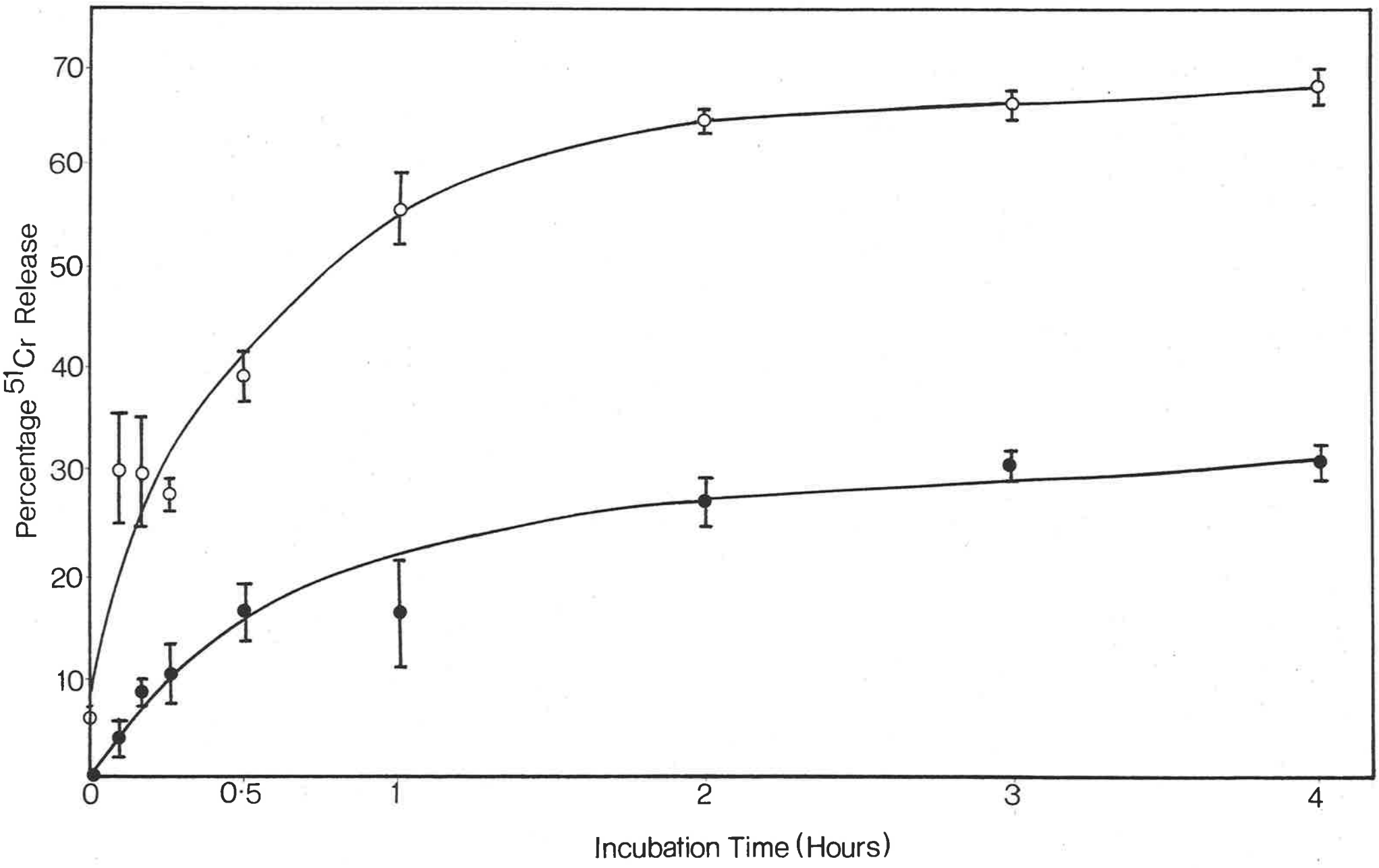
Fig. 2.3

Effect of duration of incubation in HPD

^{51}Cr-labelled Raji cells were incubated in HPD (25ug/ml) for 0-4h, resuspended in PBS and irradiated.

● 5min irradiation

O 10min irradiation


70
60
50
40
30
20
10
Percentage ^{51}Cr Release
0
0·5
1
2
3
4
Incubation Time (Hours)

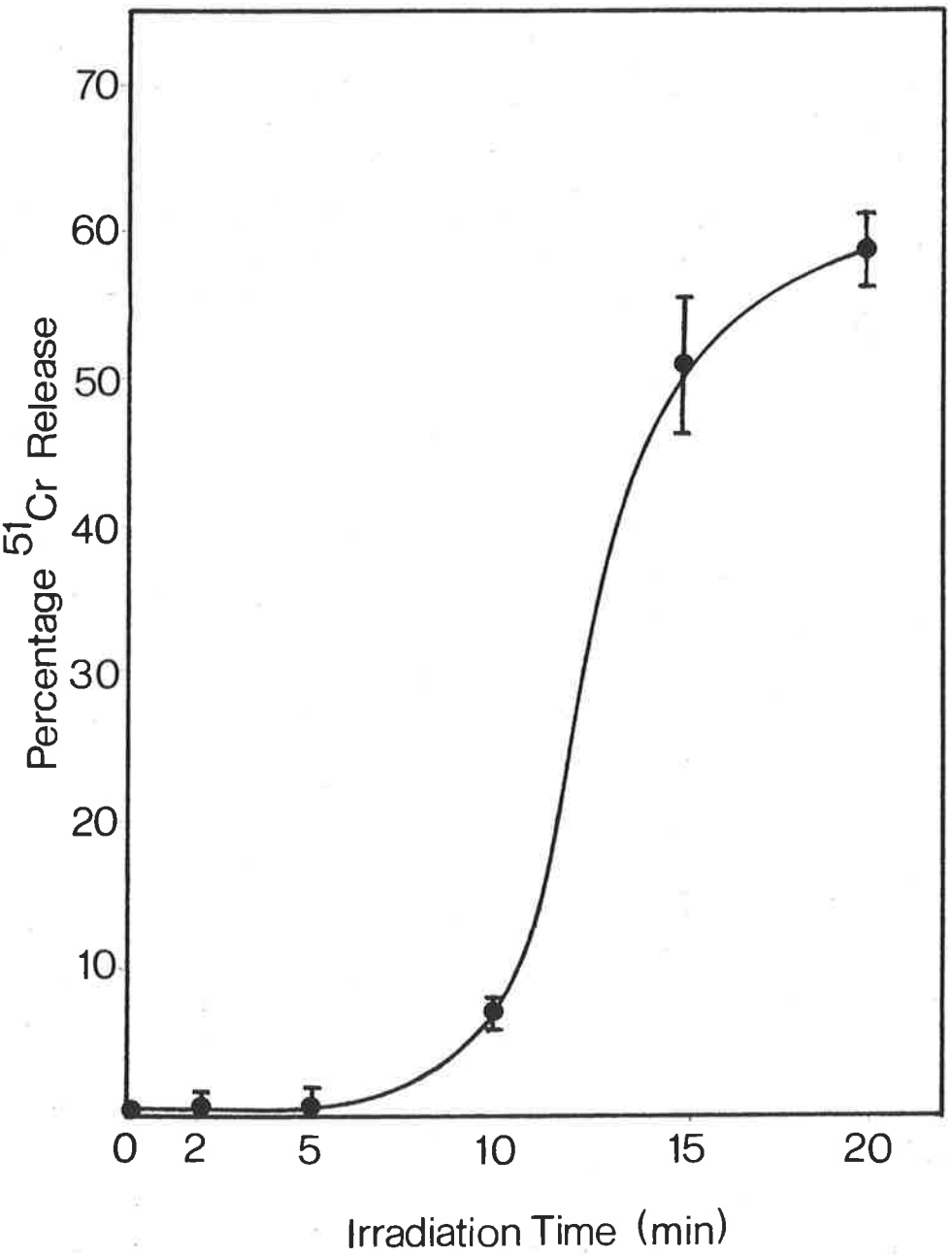


Fig. 2.4

Influence on phototoxicity of short contact time with HPD

^{51}Cr-labelled Raji cells were suspended in HPD (25ug/ml), centrifuged and immediately washed with PBS. The cells were then resuspended in PBS and irradiated.

rim of the cells by fluorescence microscopy, confirming the rapid uptake of HPD.

(c) Delayed ^{51}Cr release after irradiation

The above experiments examined ^{51}Cr release immediately after irradiation. Cell lysis is likely to be a continuous process rather than being limited to an immediate burst of ^{51}Cr release during irradiation. Delayed ^{51}Cr release was examined in the following experiment.

Method

^{51}Cr-labelled Raji cells were incubated with HPD (50ug/ml) as described above, irradiated for 2min and then incubated at 37^{o} in the dark. Aliquots of cells were removed at 1h intervals, centrifuged, and ^{51}Cr release determined as above.

Results

There was a linear increase in percentage ^{51}Cr released over the 3h following irradiation (Fig. 2.5). Irradiation for 2min caused sublethal damage to the cell since there was no ^{51}Cr release immediately after irradiation. However the ability of these cells to survive in culture was impaired.

(d) Effect of serum on ^{51}Cr release

The affinity of porphyrins for serum proteins, in particular albumin and haemopexin and the influence of serum on uptake of HPD has been considered in Chapter 1. Albumin is the main carrier of porphyrins *in vivo*, so the binding of HPD to albumin may influence the efficacy of PDT.

The effect of serum on the rate of ^{51}Cr release from HPD-sensitized Raji cells was examined. Serum was added either during HPD incubation or during irradiation.

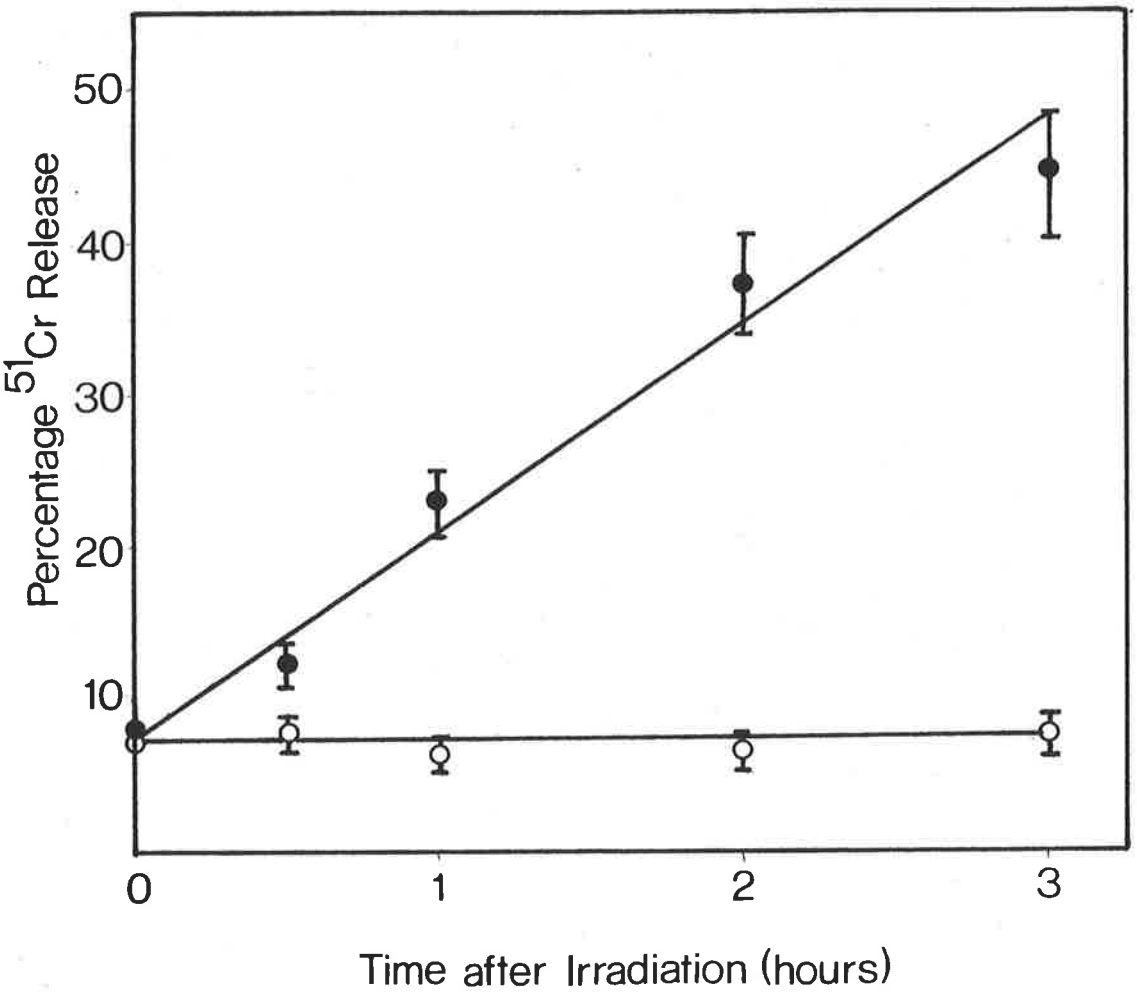


Fig. 2.5

Delayed ^{51}Cr release after irradiation

^{51}Cr-labelled Raji cells were incubated for 1h with HPD (50ug/ml), resuspended in PBS and irradiated for 2min. Control cells were not irradiated. Cells were incubated at 37^{o} in the dark and aliquots were removed at 1h intervals for determination of percentage ^{51}Cr release.

● 2min irradiation

O No irradiation

Method

^{51}Cr-labelled Raji cells (10^7/ml in RPMI 1640) were incubated for 1h at 37^{o} with HPD (150ug/ml). In some experiments, 10%FCS, 10% human serum, fetuin (500ug/ml) or bovine serum albumin (BSA, 3mg/ml) was added during incubation. Irradiations and percentage ^{51}Cr release were determined as described above. Alternatively, cells were incubated with HPD in serum-free medium and FCS, human serum, fetuin or BSA were added during irradiation in the same concentrations as above. The concentrations of fetuin and BSA were chosen to approximate those found in FCS [Holmes and Wolfe, 1961].

Uptake of HPD was assessed by fluorescence as previously described.

Results

^{51}Cr release was inhibited by adding 10%FCS during incubation with HPD (Fig. 2.6). BSA also inhibited ^{51}Cr release but fetuin did not. The most marked effect was seen with human serum. There was no ^{51}Cr release after irradiation of cells incubated in serum fractions without HPD. Irradiation in the presence of serum caused less inhibition of ^{51}Cr release than the addition of serum during incubation with HPD (Fig. 2.7). FCS inhibited ^{51}Cr release and the compound partially responsible for this effect was BSA and not fetuin.

Inhibition of ^{51}Cr release by serum could be a result of inhibition of uptake of HPD. Brighter fluorescence was observed in cells incubated in HPD in the absence of serum than with FCS, BSA or human serum added, indicating uptake of HPD was reduced in the presence of serum. Fetuin had no effect on fluorescence. Intensity of fluorescence decreased when cells

Fig. 2.6

Effect of serum on ^{51}Cr release

^{51}Cr-labelled Raji cells were incubated for 1h in HPD (150ug/ml) plus serum in the concentrations shown below. The cells were resuspended in PBS and irradiated.

● HPD only

○ HPD + 10%FCS

▼ HPD + 10% human serum

■ HPD + fetuin (500ug/ml)

□ HPD + BSA (3mg/ml)

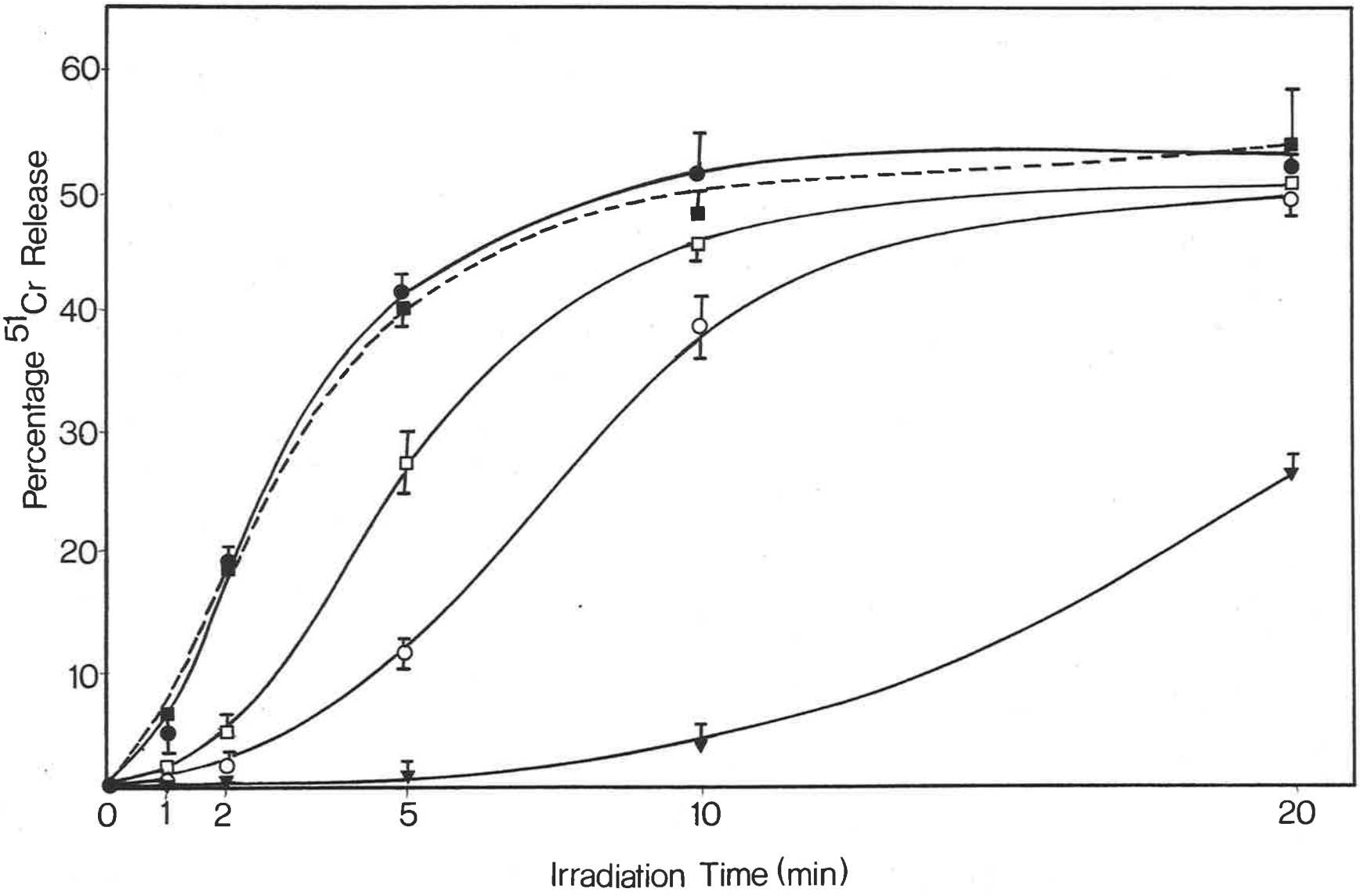
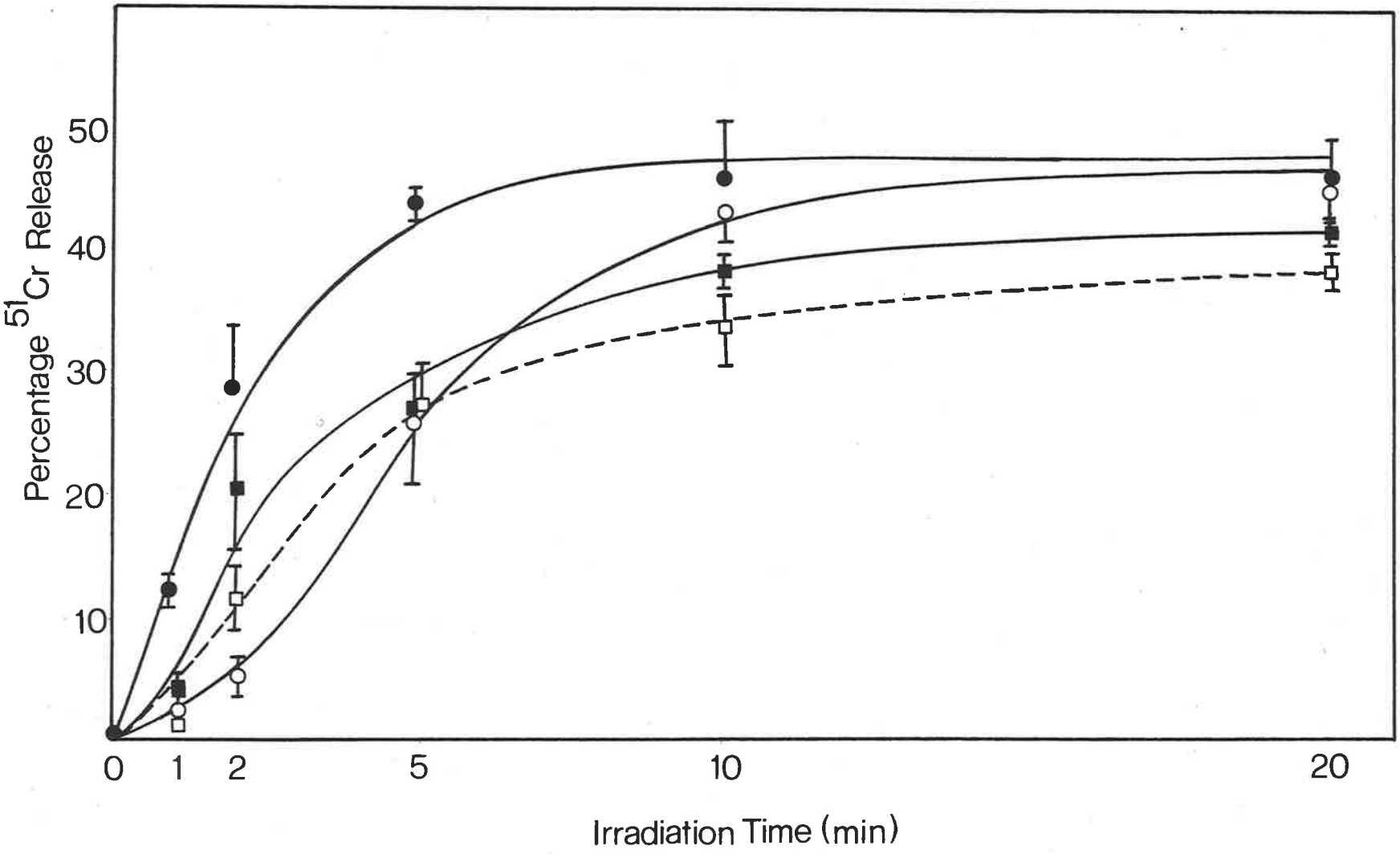

Percentage ^{51}Cr Release
60
50
40
30
20
10
0
1
2
5
10
20
Irradiation Time (min)

Fig. 2.7

Effect of serum on ^{51}Cr release

^{51}Cr-labelled Raji cells were incubated for 1h with HPD (150ug/ml) and resuspended in PBS containing serum in the concentrations described below and irradiated.

- ● HPD
- ○ HPD + 10%FCS
- ■ HPD + fetuin (500ug/ml)
- □ HPD + BSA (3mg/ml)


Percentage ^{51}Cr Release
50
40
30
20
10
0
1
2
5
10
20
Irradiation Time (min)

were suspended in serum after incubating with HPD in serum free medium, suggesting serum caused efflux of HPD from cells.

(e). ^{51}Cr Release after extended incubation with HPD followed by efflux

As discussed in Chapter 1, the mechanism of uptake and retention of HPD within cells is unclear. Uptake of HPD after extended incubation has been examined as this may be a better approximation to the in vivo situation than short incubation. The proportion of tightly bound HPD within a cell increases with extended incubation. This fraction cannot be washed out of the cells by serum-rich medium and is a more efficient photosensitizer than loosely bound HPD.

In the following experiments, ^{51}Cr release was examined after extended incubation of Raji cells with HPD followed by incubation in serum-rich medium to allow efflux of HPD.

Method

Raji cells were suspended at $0.5X10^6$/ml in RPMI 1640/10%FCS and 25ug/ml HPD. The flasks were incubated for 21h at 37^o in 5%CO_2, the supernatant removed and replaced with fresh RPMI 1640/10%FCS and ^{51}Cr (20uCi/ml). After 4h at 37^o, the cells were washed three times in RPMI 1640, resuspended at $0.5X10^6$/ml in PBS and 1ml aliquots irradiated for 0-20min. Percentage ^{51}Cr was determined immediately after irradiation as described above.

Parallel experiments were also run, incubating ^{51}Cr-labelled Raji cells in HPD (25ug/ml) in RPMI 1640/10%FCS for 1h only.

Results

^{51}Cr release was more rapid when the cells were incubated

in HPD for 21h with 4h efflux compared to 1h incubation in HPD (Fig. 2.8). Thus the former conditions resulted in more effective photosensitization of Raji cells. However the relative intracellular concentrations of HPD after the two different incubation conditions could not be measured.

(f). Relationship between ^{51}Cr release and trypan blue uptake

The uptake of vital dyes such as trypan blue has been used extensively to measure cell viability. The dye is excluded by living cells and is only taken up when the cell membrane is no longer intact [Sullivan et al., 1972]. Since both ^{51}Cr release and trypan blue uptake measure damage to cell membranes, the relationship between the two assays has been examined.

Method

^{51}Cr-labelled Raji cells were incubated for 1h in HPD (50ug/ml), then irradiated for 1-20min. Percentage ^{51}Cr release was determined as described above.

For trypan blue uptake studies, Raji cells were incubated for 1h in HPD (50ug/ml) and irradiated for 1-20min. Immediately after irradiation, 100ul Raji cells was added to 100ul of 0.3% trypan blue in PBS and examined under a phase contrast microscope. The percentage cells stained with trypan blue was counted. All assays were carried out in duplicate and the mean percentage trypan blue-positive cells calculated. The background percentage of trypan blue-positive cells (cells incubated in HPD but not irradiated) was subtracted. Cells not exposed to HPD but irradiated were also tested for viability.

Results

Untreated cells, cells treated with light only and cells

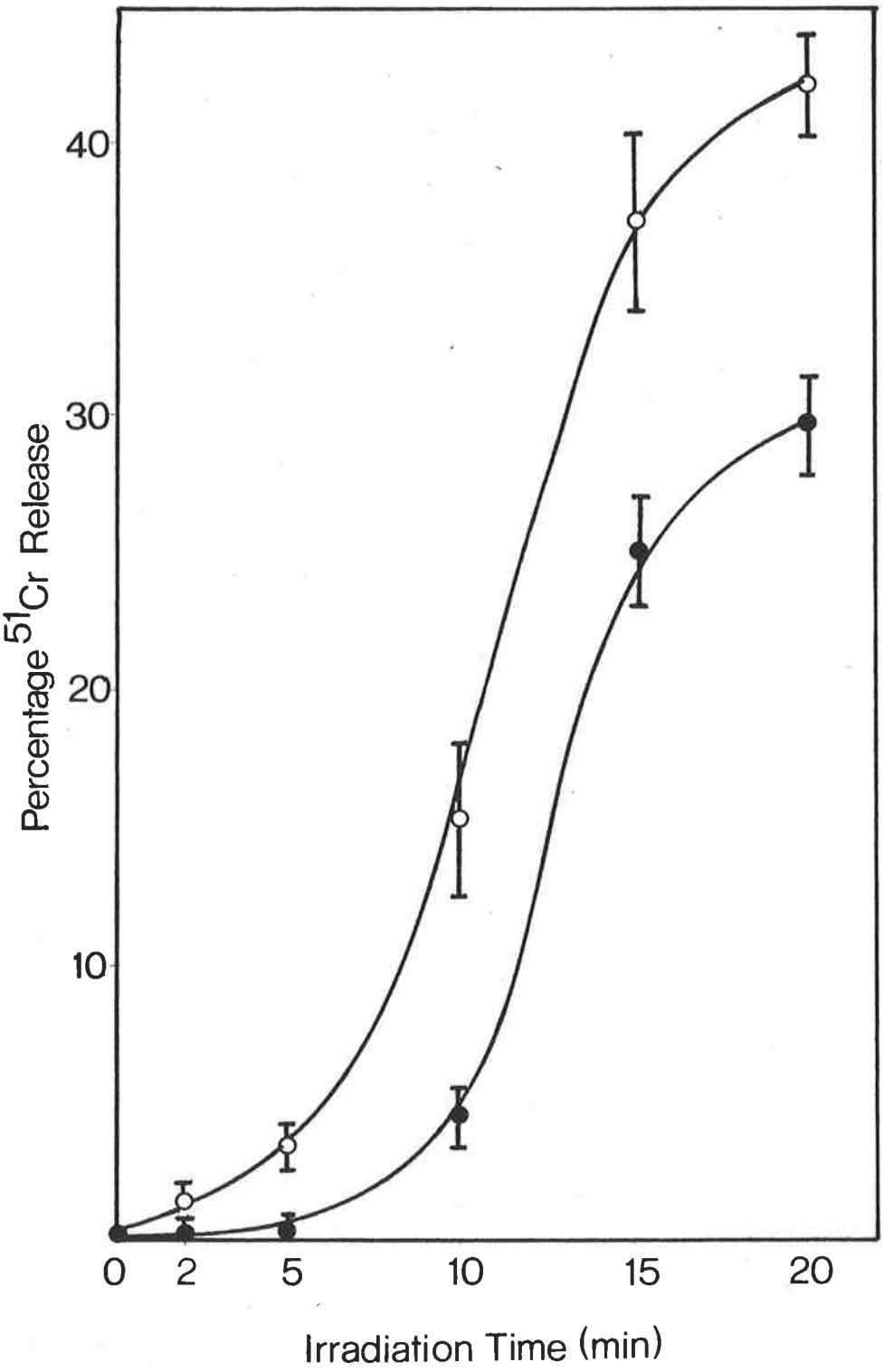
Fig. 2.8

Effect of extended HPD incubation and efflux on ^{51}Cr release

Raji cells were suspended in RPMI 1640/10%FCS with HPD (25ug/ml). One group of cells were incubated for 21h, washed and incubated for 4h in RPMI 1640/10%FCS and 20uCi/ml ^{51}Cr, then resuspended in PBS and irradiated. Alternatively, 20uCi/ml ^{51}Cr was added, the cells incubated for 1h, resuspended in PBS and irradiated.

○ Extended incubation and efflux

● Short incubation, no efflux


40
30
20
10
Percentage ^{51}Cr Release
0
2
5
10
15
20
Irradiation Time (min)

exposed to HPD but not light were all approximately 10% trypan-blue positive. After suspending Raji cells in 3% acetic acid, 100% of the cells took up trypan blue and total ^{51}Cr release was approximately 75%. The inability to reach 100% ^{51}Cr release when all the cells are dead (100% trypan blue-positive cells) is probably due to ^{51}Cr being trapped in the cell debris and being centrifuged to the bottom of the tube. ^{51}Cr may also stick to the sides of the plastic tube.

Trypan blue uptake and ^{51}Cr release showed nearly identical profiles over the linear portion of the graph when cell destruction is occurring (Fig. 2.9). Therefore ^{51}Cr release and trypan blue uptake may be measuring the same parameters of damage to the cells.

(g). Inhibition of ^{3}H Thymidine Uptake by HPD and Light.

Incorporation of ^{3}H-thymidine into the DNA of dividing cells has been used as a measure of cell viability, and more particularly of the reproductive capacity of the cell [Roper and Drewinko, 1976, 1979; Moan et al., 1983a]. The capacity of HPD and light to inhibit cell division was investigated. The relationship between inhibition of ^{3}H-thymidine uptake and other parameters of cell damage, measured by ^{51}Cr release or trypan blue uptake was also examined.

Method

Raji cells (10^{7}/ml in RPMI 1640/10%FCS) were incubated for 1h with HPD (50ug/ml). The cells were washed, resuspended at 10^{6}/ml in RPMI 1640/10%FCS and 1ml aliquots were irradiated for 0-20min. Triplicates (200ul) were incubated with 1uCi ^{3}H-thymidine in flat bottomed microculture plates (Linbro) for 18h at 37^{o} in 5%CO_2. Cultures were collected onto glass fibre discs by cell harvester (Skatron), the discs suspended in toluene

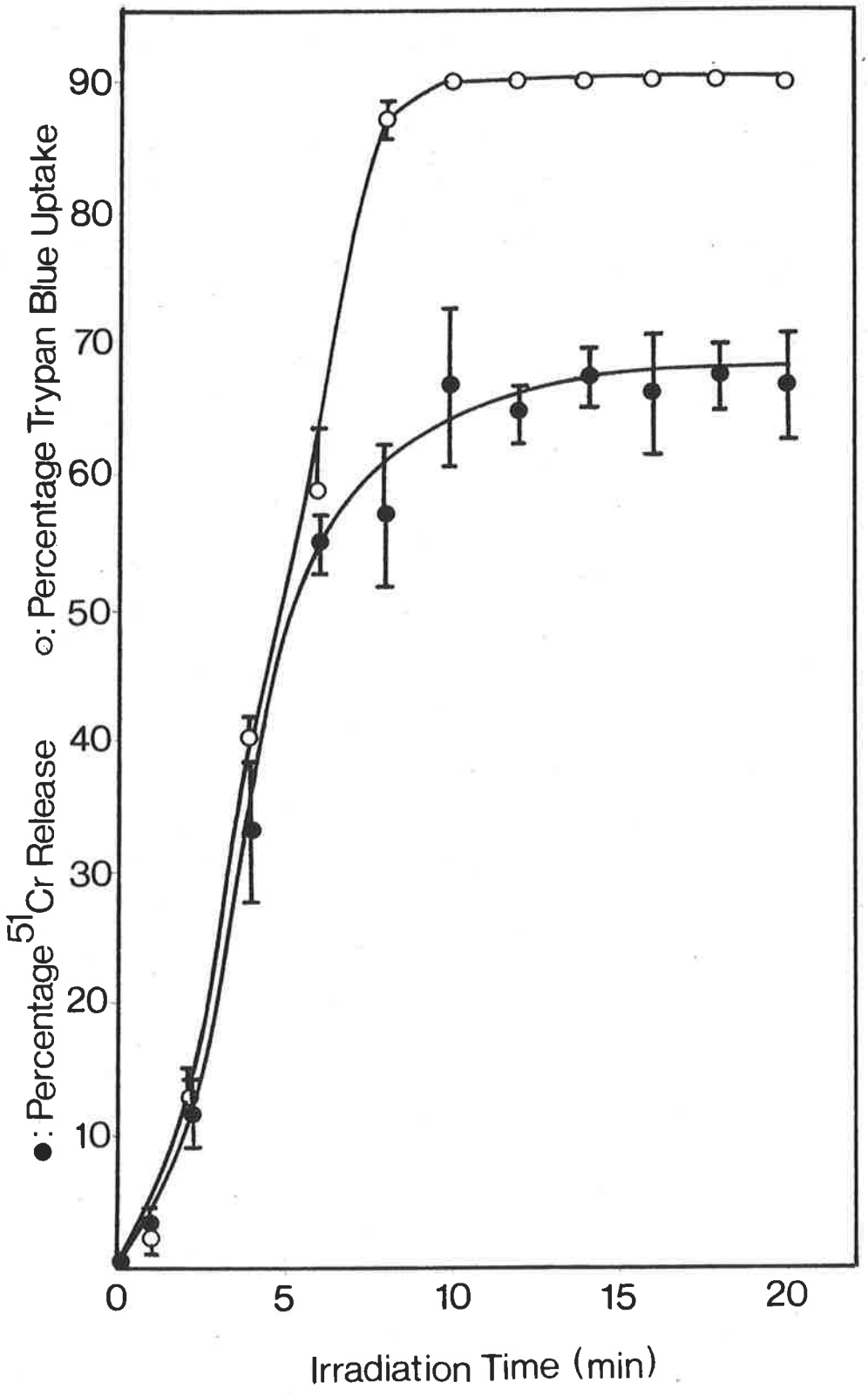


Fig. 2.9

Comparison between ^{51}Cr release and trypan blue uptake

Raji cells were incubated for 1h in HPD (50ug/ml), resuspended in PBS and irradiated. Trypan blue uptake or ^{51}Cr release were determined immediately after irradiation.

scintillation fluid containing 0.4% w/v 2,5-diphenlyoxazole plus 0.03% w/v 1,4-di[2-(5-phenyloxazolyl)] benzene and counted in a Beckman LS2800 liquid scintillation counter. Results were obtained in counts per minute. Means of the triplicates and the percentage inhibition of ^{3}H-thymidine uptake were calculated, taking as 100%, uptake in cells incubated with HPD but not exposed to light.

In parallel experiments, ^{51}Cr release was measured as described above. In some experiments, percentage ^{51}Cr release was measured immediately after irradiation. Alternatively, irradiated cells were incubated for 18h at 37^{o} before determination of ^{51}Cr release.

Results

Inhibition of ^{3}H-thymidine uptake in HPD-sensitized Raji cells was observed after 1min irradiation followed by increased inhibition until a plateau was reached (Fig. 2.10). Standard deviations between triplicate cultures were less than 10% and less than 15% between duplicate experiments. There was no inhibition of ^{3}H-thymidine uptake in cells sensitized with HPD but not irradiated, or in cells exposed to light but not incubated in HPD.

^{51}Cr release and inhibition of ^{3}H-thymidine uptake were compared in Fig. 2.11. There was no ^{51}Cr release immediately after irradiation, indicating that the cells were still intact. After incubating the cells for 18h, ^{51}Cr release paralleled the inhibition of ^{3}H-thymidine uptake. There was a linear relationship between percentage ^{51}Cr release and percentage inhibition of ^{3}H-thymidine uptake (Fig. 2.12).

Fig. 2.10

Inhibition of ^{3}H thymidine uptake by HPD and light

Raji cells were incubated for 1h in RPMI 1640/10%FCS with HPD (50ug/ml), resuspended in RPMI 1640/10%FCS and irradiated. Cells were incubated for 18h at 37^{o} with 20uCi ^{3}H-thymidine before harvesting.

● HPD (50ug/ml)

O No HPD

Fig. 2.11

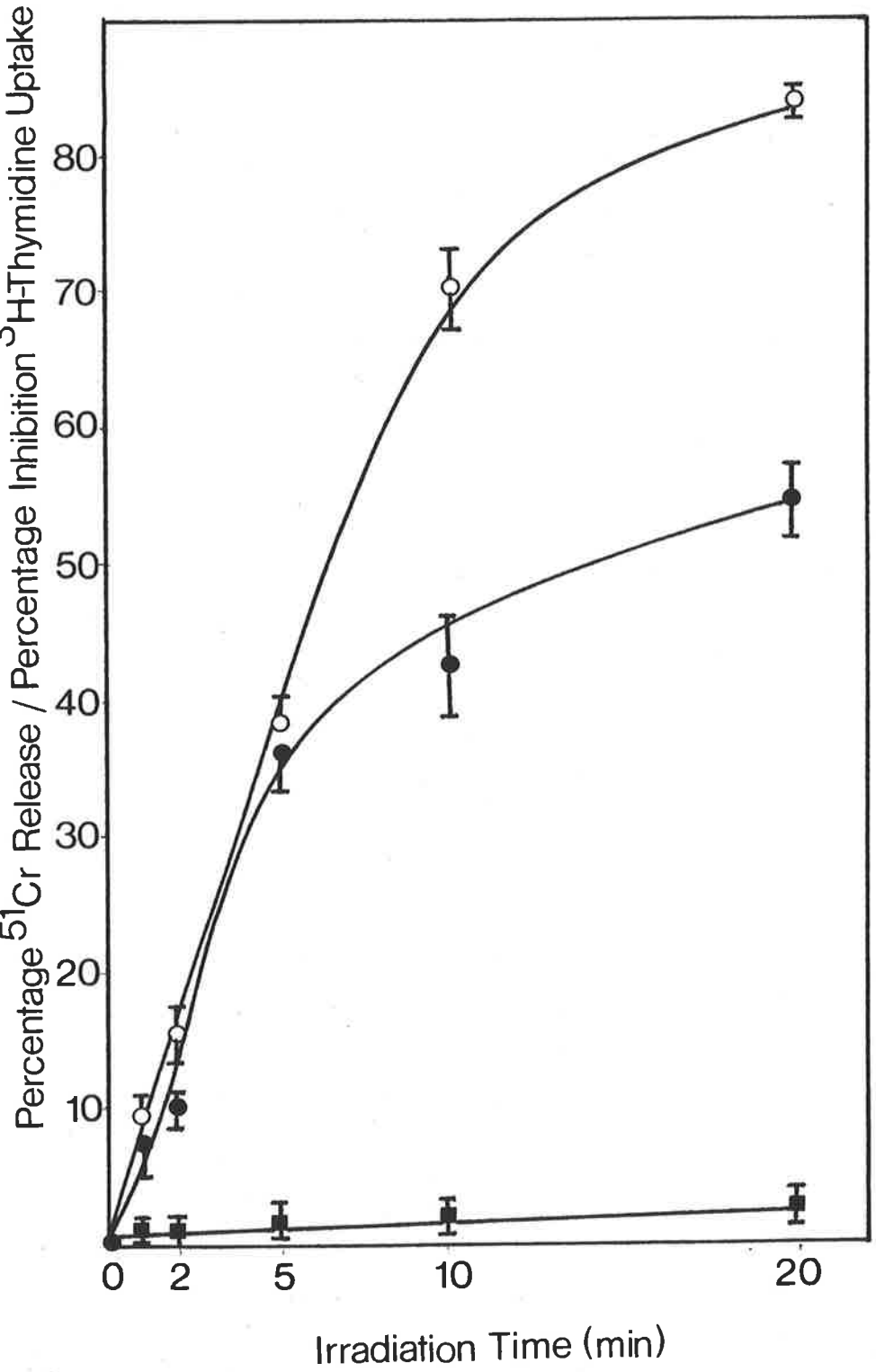
Relationship between percentage ^{51}Cr release and percentage inhibition of ^{3}H thymidine uptake

Raji cells were incubated for 1h in RPMI 1640/10%FCS with HPD (50ug/ml), resuspended in RPMI 1640/10%FCS and irradiated. The cells were incubated for 18h at 37^{o} and percentage ^{51}Cr release and percentage inhibition ^{3}H-thymidine uptake determined.

■ Percentage ^{51}Cr release immediately after irradiation

● Percentage ^{51}Cr release 18h after irradiation

O Percentage inhibition of ^{3}H-thymidine uptake


Percentage ^{51}Cr Release / Percentage Inhibition ^{3}H-Thymidine Uptake
80
70
60
50
40
30
20
10
0
2
5
10
20
Irradiation Time (min)

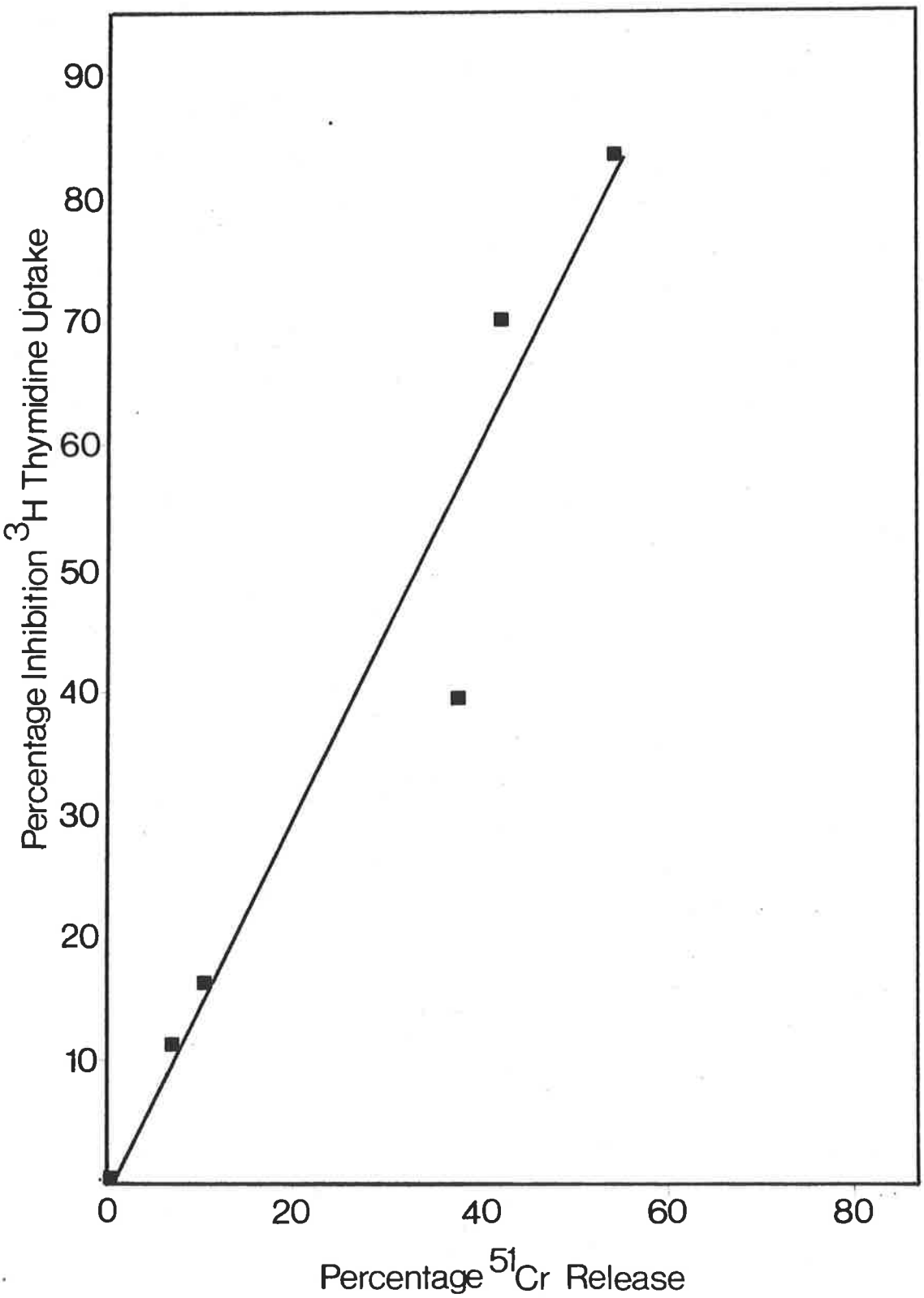


Fig. 2.12

Relationship between percentage ^{51}Cr release and inhibition of ^{3}H-thymidine uptake

The percentage ^{51}Cr release was plotted against percentage ^{3}H-thymidine uptake using the data from Fig. 2.11.

(h). Inhibition of Raji Cell Colony Growth by HPD and Light

The effect of HPD and light upon the growth of Raji cell colonies on semisolid agar has also been examined. There was gradual ^{51}Cr release over several hours after irradiation (Fig. 2.5), suggesting ^{51}Cr release measured immediately after irradiation may not accurately reflect photodynamic damage to cells. Inhibition of colony formation may be a more sensitive indicator of photodynamic damage than ^{51}Cr release. Colony formation has been used extensively to measure photocytotoxic damage to cells by porphyrins [Christensen and Moan, 1979; Moan et al., 1979; 1980a; Gomer and Smith, 1980]. Growth of colonies was inhibited after treatment of cells with HP or HPD and light. Colony formation has been used to examine the effects of temperature and pH changes on photodynamic damage to cels [Moan and Christensen, 1979].

Methods

Raji cells (10^7/ml in RPMI 1640/10%FCS) were incubated with HPD (25ug/ml) for 1h at 37^{o}. The cells were washed, resuspended at 10^6/ml in RPMI 1640/10%FCS, then irradiated with red light (630nm) in a Varian series 634 spectrophotometer for 1 to 20min. The light intensity was not measured. After diluting to $5X10^4$/ml in RPMI 1640/10%FCS, 1ml cells was poured onto 0.5% agar in RPMI 1640/10%FCS in 35X10 mm plastic petri dishes. Triplicate cultures for each irradiated tube were plated out. Cells were kept in the dark where possible during all manipulations. Cultures plates were incubated for 10 days at 37^{o}in 7.5%CO_2. Colonies containing more than 10 cells were scored under a Zeiss binocular microscope (40X magnification).

Results

The inhibition of colony formation after irradiation of

HPD-sensitized cells is shown in Fig. 2.13. Each point represents the mean value of duplicate tubes. Despite rigorously excluding room light, there was marked inhibition of colony formation by cells sensitized with HPD but not irradiated. These cells excluded trypan blue immediately after incubating with HPD but were unable to form colonies. There was further inhibition of colony formation when HPD-sensitized cells were irradiated. The colonies grown by HPD-sensitized cells were smaller, containing 10-20 cells, while control colonies contained up to 200 cells.

There was a linear relationship between the density of cells plated out and the number of colonies grown in the absence of HPD (Table 2.1). Therefore, the number of colonies was independent of the cell density, within the range tested.

(i). Discussion

This chapter examines ^{51}Cr release as an assay suitable for measuring photocytotoxicity *in vitro* of HPD and other porphyrins. The assay was reproducible, relatively rapid and capable of screening several porphyrins at once. The assay was also suitable for examining the effects of other conditions such as pH and temperature changes on cell damage and testing the interactions of PDT with other drugs. It has also been used to test the requirement of the phototoxic process for oxygen [Lee See et al., 1984].

When Raji cells were incubated with HPD and irradiated with red light, there was a linear release of ^{51}Cr with increasing HPD or light dose. A plateau was reached when all the cells had died. This linear relationship was seen by other authors [Gomer and Smith, 1980; Christensen and Moan, 1979;

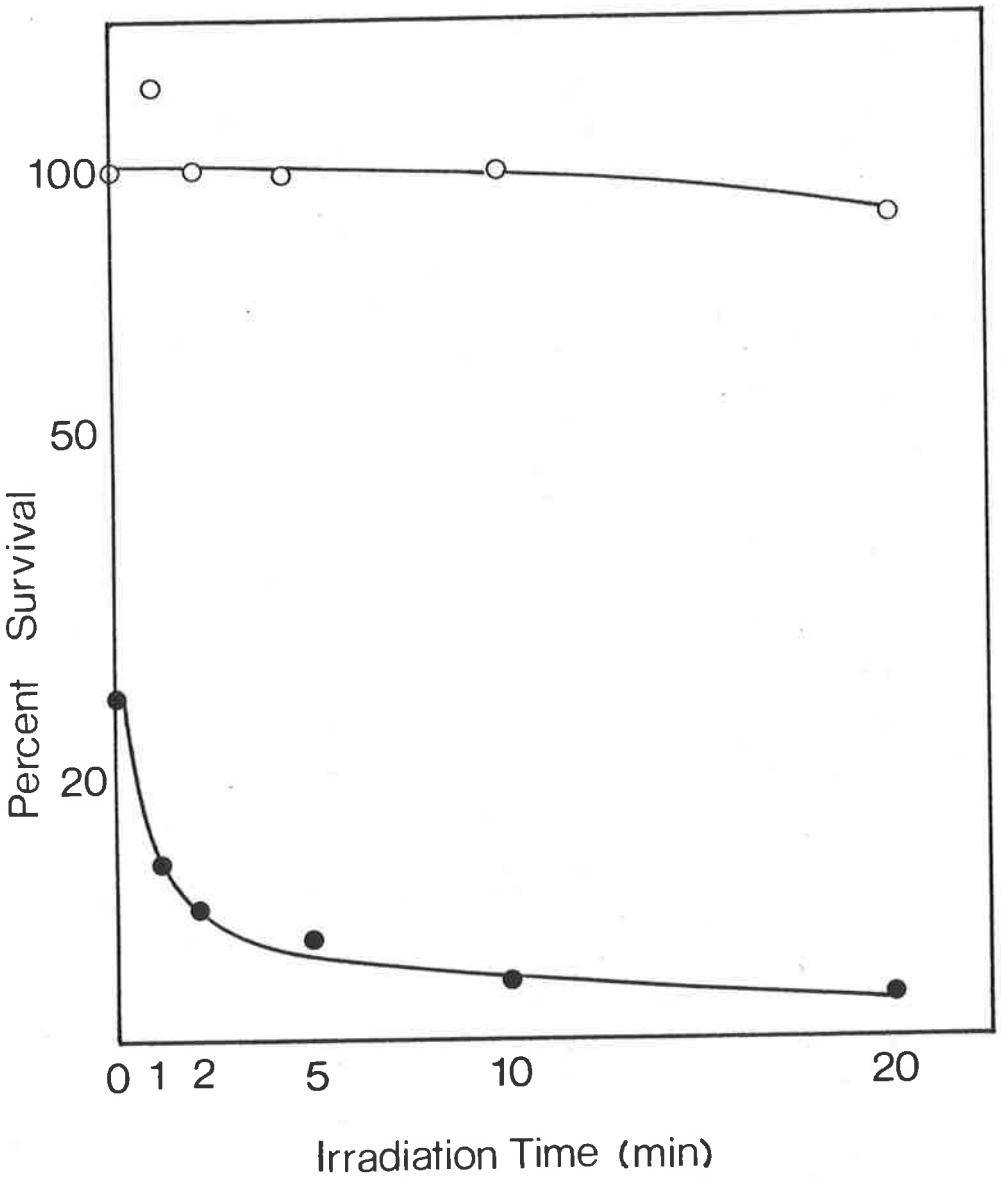


Fig. 2.13 (amended)

Influence of HPD and light on the growth of Raji cell colonies

Raji cells were incubated for 1h in RPMI 1640/10%FCS with HPD (25ug/ml), resuspended in RPMI 1640/10%FCS and irradiated. Cells were plated out onto 0.5% agar and incubated for 10 days. The number of colonies containing greater than 10 cells was scored and percentage survival calculated, taking as 100%, growth of cells without either HPD or light treatment.

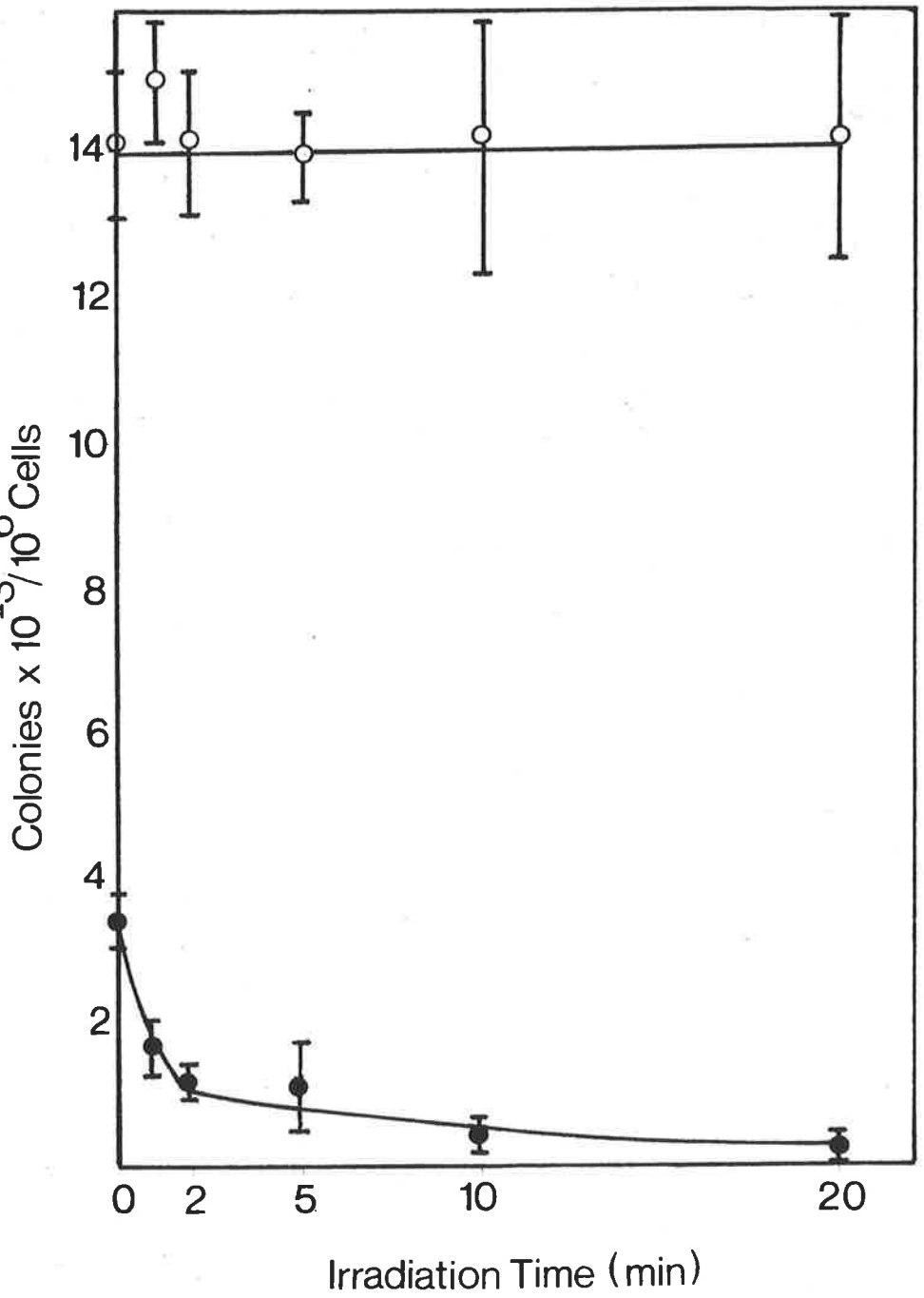


Fig. 2.13

Influence of HPD and light on the growth of Raji cell colonies

Raji cells were incubated for 1h in RPMI 1640/10%FCS with HPD (25ug/ml), resuspended in RPMI 1640/10%FCS and irradiated. Cells were plated out on 0.5% agar and incubated for 10 days. The number of colonies containing greater than 10 cells was scored.

● HPD (25ug/ml)

○ No HPD

Table 2.1

Relationship between concentration of cells plated out and number of colonies

Cell density/ml	No. Colonies/Plate	Av. No. Colonies/10^6 cells
10^4	110	11,600
2×10^4	220	11,000
4×10^4	572	14,300
5×10^4	580	11,700
7×10^4	Confluent Colonies	
10^5	Confluent Colonies	

Bellnier and Dougherty, 1982]. Bellnier and Dougherty [1982] reported that an increase in intracellular HPD concentration resulted in a corresponding decrease in the light dose required for a given level of survival. Thus the product of HPD concentration and light dose is constant for a given level of cell destruction. The degree of cell destruction is dependent on the total light dose and not the dose rate at non-thermal levels [Gomer et al., 1985b]. There was a threshold dose of HPD or light below which there was no ^{51}Cr release. The lag before ^{51}Cr release begins suggests an accumulation of damage is required for cell death. Moan et al. [1979] suggested this lag period reflected sublethal damage, during which repair of the cell was possible.

The correlation between trypan blue uptake and ^{51}Cr release indicated ^{51}Cr release occurred as a result of membrane damage to the cells. The dye was excluded by living cells and was only taken up through non-intact membranes [Wigzell, 1965; Sullivan et al., 1972]. This implies that one of the consequences of irradiating HPD-sensitized cells is membrane damage. Bellnier and Dougherty [1982] have shown a correlation between ^{51}Cr release, inhibition of colony formation and membrane lysis. As discussed in Chapter 1, membrane damage may be a late event in the photodynamic damage to HPD-sensitized cells rather than the initial site of damage. The ^{51}Cr release assay does not provide any information on the nature of the initial sites of damage in the cell.

Percentage ^{51}Cr release was reproducible within 10% within an assay. However there was considerable day to day variation and the photosensitivity of the cells altered considerably. This may be due to variations in the physiological state of the

cells, which may result in different intracellular concentrations of HPD. The capacity of the cell to repair sublethal damage may also vary. The stage of the cell cycle may also affect the photosensitivity of the cells. As discussed in Chapter 1, there are conflicting reports of the influence of the cell cycle on the susceptibility of the cells to photodynamic damage. This may be a reflection of the different cell types tested. It is not known if Raji cells differ in photosensitivity through the cell cycle. Cell cycle synchronisation was not attempted, although cells were routinely harvested when approaching stationary phase.

The influence of serum on ^{51}Cr release was examined. There was a marked inhibition in ^{51}Cr release when FCS was added either during HPD incubation or during irradiation. Intensity of fluorescence of porphyrins in the cell was reduced by FCS, implying a reduction in the uptake of HPD. This was in agreement with several authors who have shown that as little as 1% serum resulted in marked inhibition in the uptake and retention of HPD *in vitro* [Henderson et al., 1983; Moan and Christensen, 1979; 1981]. Protein-bound porphyrins were not taken up into NHIK 3025 cells and the phototoxic damage was reduced proportionally to the reduction in uptake of porphyrin [Moan and Christensen, 1981]. The above experiments showed albumin and not fetuin inhibited uptake of HPD. This would be expected since a high affinity binding between albumin and HPD and other porphyrins has been reported [Lamola et al., 1981]. Albumin may be important *in vivo* in the transport of HPD and its role in mediating the phototoxic process should be further examined. Recent studies have also shown a strong affinity

between HPD and lipoproteins, particularly low density lipoproteins (LDL) [Kessel et al., 1986], so LDL may also be important in the transport and uptake of HPD.

Porphyrin was eluted from cells after washing in serum-rich medium [Bellnier and Lin, 1983; Henderson et al., 1983; Moan et al., 1984]. With increasing incubation time, an greater proportion of tightly bound HPD was not washed out of the cells by serum. This fraction was a more efficient photosensitizer. The results above showed that extended incubation in HPD, followed by efflux, resulted in greater ^{51}Cr release then 1h incubation in HPD. However, it was not possible to measure intracellular HPD concentrations and these may be different.

The inhibition of ^{3}H-thymidine uptake and colony formation after irradiation of HPD-sensitized Raji cells were also examined and the relationship of these parameters to ^{51}Cr release was considered. Irradiation of HPD-sensitized Raji cells resulted in inhibition of ^{3}H-thymidine uptake and colony formation that was proportional to the light dose. Moan et al. [1983a] reported inhibition of ^{3}H-thymidine uptake in HPD-sensitized NHIK 3025 cells after irradiation. They found ^{3}H-thymidine uptake was more sensitive in detecting cell damage than ^{51}Cr release. This agrees with the above results. Inhibition of ^{3}H-thymidine uptake occurred in the absence of ^{51}Cr release measured immediately after irradiation. However the cells were damaged and gradually died over 18h (shown by delayed ^{51}Cr release).

The division and growth of Raji cells was inhibited by treatment with HPD and light, as demonstrated by inhibition of both ^{3}H-thymidine and colony formation. The reduced size of colonies suggested that division of surviving cells was

inhibited. HPD alone caused marked inhibition of colony formation, but had no effect on ^{3}H-thymidine uptake, ^{51}Cr release or trypan blue uptake. The small amount of room light to which the cells were exposed may have caused photodynamic damage, but HPD alone may have slight cytotoxic effects. Malik and Djaldetti [1980a] reported PP was cytotoxic to human lymphocytes and leukaemic cells without irradiation. Berns et al. [1983] also inhibited the growth of PTK2 kidney cells by treating with HPD in the dark.

The results in this chapter suggested that colony formation was the most sensitive indicator of photodynamic damage to Raji cells. However the assay was difficult to perform, was less reliable and was not amenable to screening large numbers of samples. ^{51}Cr release, while not as sensitive as colony formation or ^{3}H-thymidine uptake, had the same relationship between HPD and light dose and cell inactivation as the other methods. Thus it was decided to use the ^{51}Cr release assay routinely for testing photocytotoxicity in vitro.

CHAPTER 3

IN VIVO MEASUREMENT OF PHOTOCYTOTOXICITY USING A TRANSPLANTABLE TUMOUR MODEL IN MICE

(a). Introduction

The previous chapter described an assay to measure the photocytotoxic activity of HPD *in vitro*. This assay examined the direct interaction between tumour cells and HPD but could not take into account other interactions that may be important *in vivo*. The mechanisms of transport and delivery of HPD to the tumour cannot be studied *in vitro*. Most of the circulating porphyrins are bound to albumin [Morgan et al., 1980; Lamola et al., 1981] or to LDL [Kessel et al., 1986]. Serum proteins and lipoproteins will be important in delivering porphyrin to the tumours and will influence efficacy of treatment.

The greatest HPD concentration is in the vascular stroma of tumours rather than in the tumour cells [Bugelski et al., 1981]. As discussed in Chapter 1, damage to the microvasculature may indirectly cause tumour necrosis by infarction [Henderson et al., 1985; Star et al., 1984]. Therefore the efficacy of photodynamic destruction of the tumour cells may not be the major factor in determining efficacy of tumour destruction. All these mechanisms require study *in vivo*. The therapeutic efficacy of a particular porphyrin may result from a number of these interactions. Other manipulations that could result in a more effective tumour response, such as the concurrent administration of other drugs may also be studied *in vivo*.

Therefore an assay to measure photoactivity *in vivo* was developed using transplantable mouse tumours. Lewis lung

carcinoma [Sugira and Stock, 1955], has been used extensively in cancer research for examining efficacy of chemotherapeutic drugs [Karrer et al., 1967; Mayo, 1972]. It has also been used to investigate mechanisms of metastasis [Trope, 1975; Donelli et al., 1979; Fogel et al., 1979]. B16 melanoma has also been used to screen chemotherapeutic agents [Griswold, 1972]. Both Lewis lung carcinoma and B16 melanoma were tested for response to PDT.

(b). Methods

(i). Mouse tumour assay

Inbred C57Bl/6J mice of either sex were obtained at approximately 8 weeks of age from the Department of Agriculture, Animal Resource Centre, Adelaide. Mice with Lewis lung carcinoma or B16 melanoma growing subcutaneously in the back were obtained from Mr. L. Dent, Department of Microbiology, Flinders Medical Centre. The mice were killed by cervical dislocation, the tumours dissected out, chopped finely and the fragments passed through a fine wire sieve to give a single cell suspension. Cell viability was approximately 50% (trypan blue uptake). The concentration was adjusted to $10X10^6$/ml viable cells in RPMI 1640 and 100ul injected subcutaneously into the back of the mouse. In later experiments, Lewis lung carcinoma cells were maintained in culture in RPMI 1640/10%FCS, 2.3mM $NaHCO_3$, 25mM HEPES and 0.16ug/ml gentamicin. The cells were harvested by treatment for 1min with 0.01% trypsin in PBS and resuspended at $10X10^6$/ml in RPMI 1640 for transplanting into mice as above. Fresh cultures were established every three months from stock frozen in liquid nitrogen. After 7-10 days, both Lewis lung carcinoma and B16

melanoma were approximately 5-7mm in diameter and ready for treatment. There was no difference in the growth rate or efficiency of transplantation between mice transplanted with Lewis lung carcinoma cells grown in culture or removed from a mouse.

The assay was modified from Dougherty et al. [1983]. Mice in groups of ten were given HPD (25-60mg/kg i.v. or i.p.). Twenty four hours later, the mice were anaesthetised with 60mg/kg sodium pentobarbitone (Sagatal, May and Baker Aust. Pty. Ltd), the fur over the tumours shaved and a 1cm diameter spot over the tumours irradiated with an appropriate dose of red light. Mice were examined daily for the presence of palpable tumour. After an effective treatment, tumours were impalpable twenty four hours after irradiation. The end point of the assay was the number of days for 5 out of 10 tumours to recur (TC_{50}). All assays were performed in duplicate and the means and standard deviations calculated.

(ii). Skin photosensitivity

Mice were injected with HPD (50mg/kg i.p.) and the left footpad of the hind leg was irradiated 24h later with 110J/sq cm from the incandescent lamp. The control right foot was untreated. Twenty four hours after irradiation, footpad thickness was measured using a micrometer gauge. The percentage increase in thickness of the left foot compared to the right was calculated.

(iii). Light sources

A gold metal vapour laser, designed and built by Quentron Optics Pty. Ltd., Adelaide, delivered pulsed monochromatic light at a wavelength of 627.8nm. The pulse width at base was

50ns and the pulse repetition frequency was 10-14kHz. The laser beam was coupled to a 400u quartz fibre, and positioned to create a 1cm diameter spot.

A Spectra Physics 164 Argon Ion laser pumping a Rhodamine B dye laser was used as a source of continuous wave red laser light. It was operated without the tuning wedge and the output was 300-500mW over the wavelength 625-635nm. The beam was coupled to a 400u quartz fibre which was used to create a 1cm diameter spot.

An incandescent beam lamp was designed by Dr. F. Jacka and Dr. A.J. Blake, Department of Physics and The Mawson Institute, The University of Adelaide [Jacka and Blake, 1983]. A tungsten-halogen lamp with a total output of 1000W was fitted with appropriate short and long pass filters to deliver light of wavelength 610-680nm. The lamp was fitted with a perspex lens 10mm in diameter and 50mm length and delivered 2.5W uniformly over a 1cm diameter spot. The effective light flux at 630nm was 890mW [Wilksch, 1982, unpublished data]. Because of the high power density, the skin of the mouse was cooled with a fine spray of water and irradiated in 50sec exposures with 10sec pauses.

(iv). Tumour fluorescence

Tissues were removed from mice 24h after administration of HPD (50mg/kg i.p.), snap frozen on dry ice and stored at -80^{o}. Sections (6u) were cut at -14^{o} on an IEC microtome and examined immediately for red fluorescence under a Zeiss fluorescence microscope with excitation wavelength 420-490nm.

(v). Histology

Histological sections were prepared by the Histopathology Department, The Queen Elizabeth Hospital. Mice were given HPD

(50mg/kg i.p.), followed 24h later by irradiation of the tumours with 225J/sq cm light. Tumours were removed 24h after irradiation and fixed in formalin. Paraffin sections were prepared and stained with haematoxylin and eosin by standard methods.

(c). Relationship between TC_{50} and HPD dose

Mice with Lewis lung carcinoma were given HPD (25-60mg/kg i.p) and the tumours irradiated 24h later with 200sec light (225J/sq cm) from the incandescent lamp. There was a linear relationship between the HPD dose and the time to 50% recurrence (TC_{50}) (Fig 3.1). Control mice were injected with HPD only, irradiated with light only or received no treatment. There was no reduction in size of the tumours, indicating that both HPD and light were necessary for tumour regression.

The experiment was then repeated administering HPD (25-60mg/kg) intravenously. This was more efficient in causing tumour necrosis than intraperitoneal HPD, as the TC_{50} was one day greater for all doses tested (Fig. 3.1). There was also a linear relationship between light dose and TC_{50}. Since the difference was not large, i.p. injections were used routinely.

(d). Relationship between TC_{50} and light dose

Mice with Lewis lung carcinoma were given HPD (50mg/kg i.p.) and irradiated 24h later with either the incandescent lamp (110-340J/sq cm), the gold laser (100-250J/sq cm) or the Argon ion/dye laser (110-250J /sq cm). There was a linear relationship between TC_{50} and light dose for all three light sources (Fig. 3.2). The two lasers and the incandescent lamp were all equally efficient in causing tumour destruction.

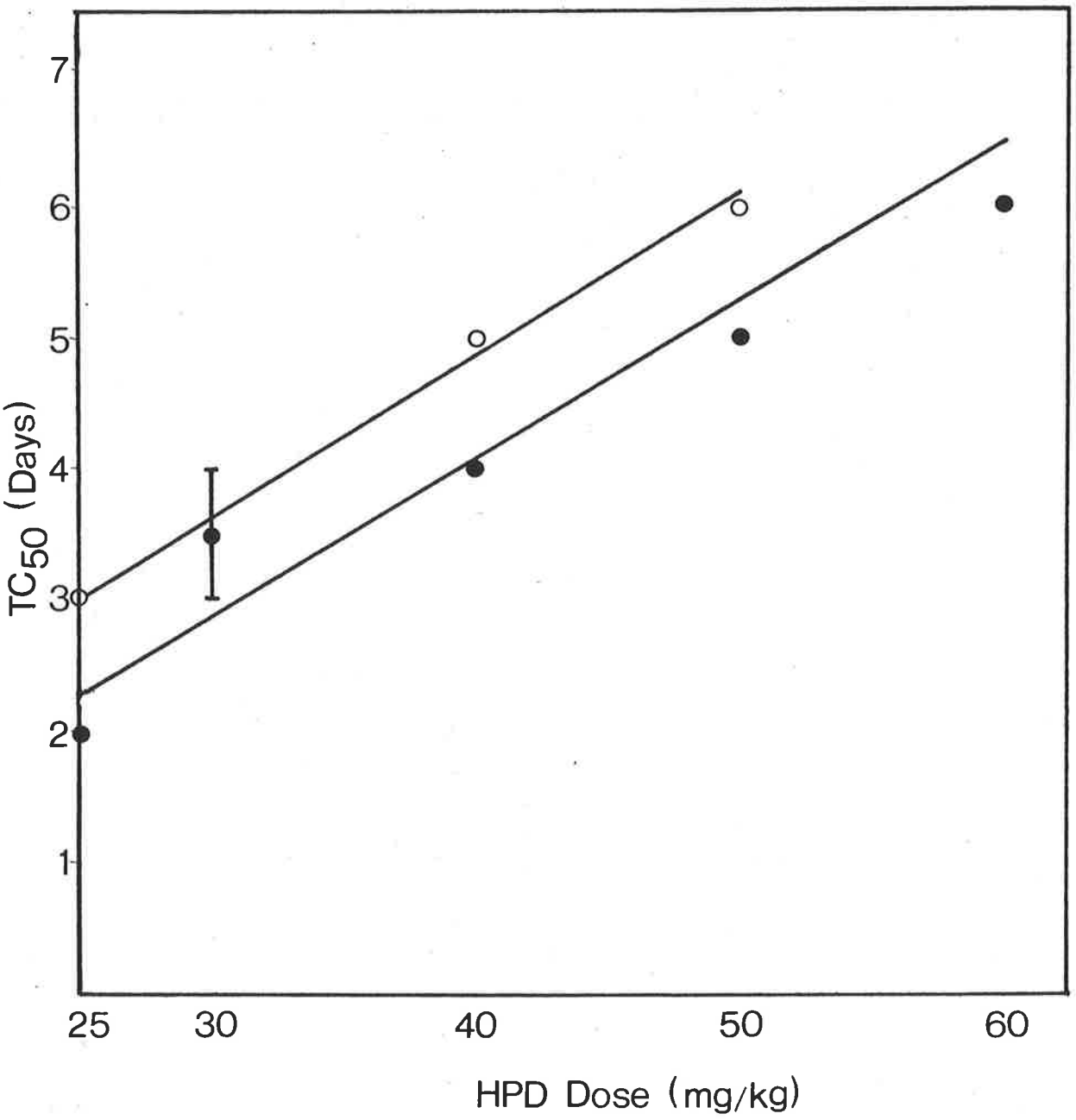


Fig. 3.1

Relationship between TC_{50} and HPD dose

Mice with Lewis lung carcinoma were given HPD (24-60mg/kg i.v. or i.p.) and irradiated 24h later with 225J/sq cm light.

○ HPD administered i.v.

● HPD administered i.p.

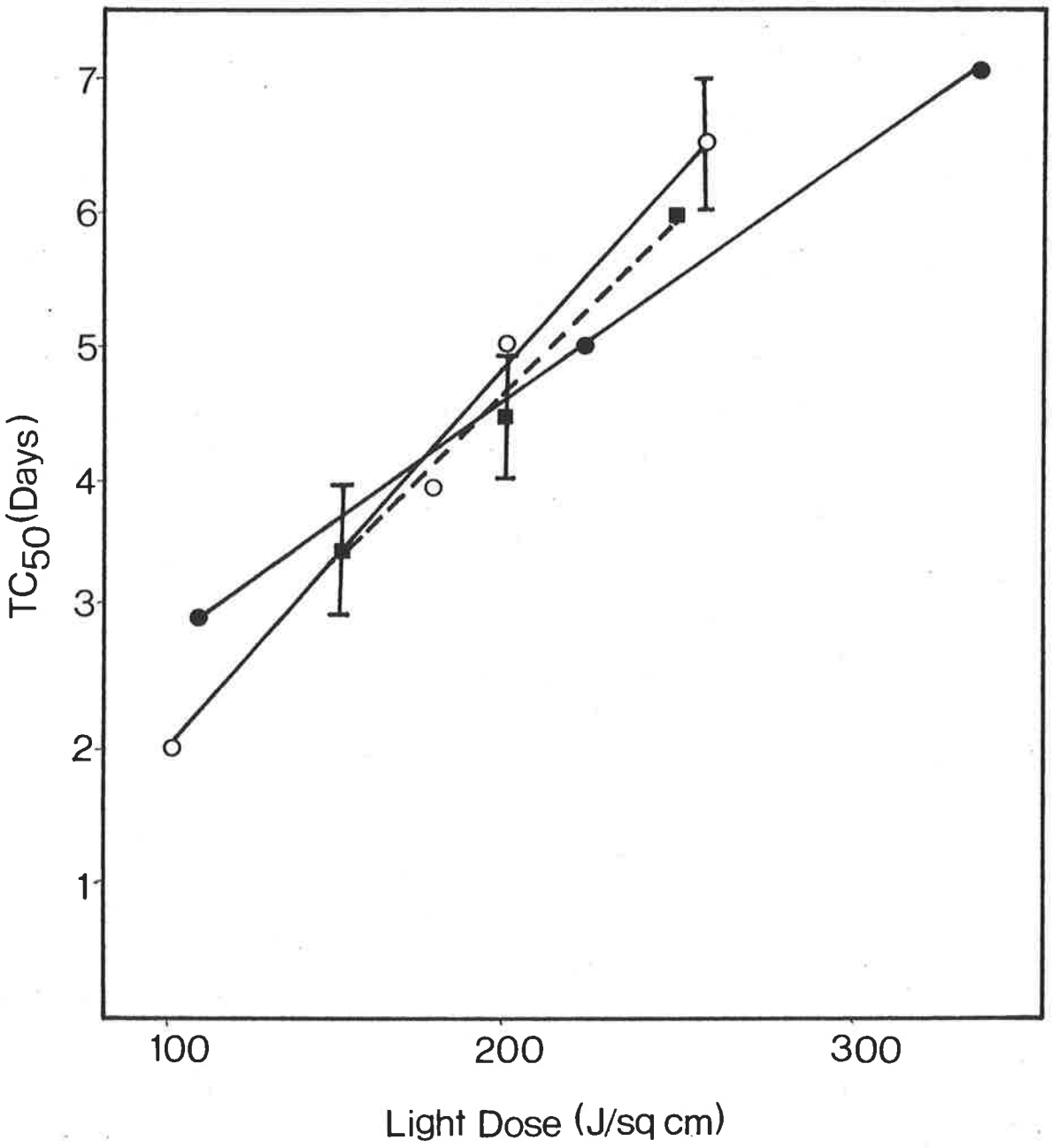


Fig. 3.2

Relationship between TC_{50} and light dose

Mice with Lewis lung carcinoma were given HPD (50mg/kg i.p.) and irradiated 24h later with 100-340J/sq cm light.

- ● Incandescent lamp (610-680nm)
- O Gold laser (pulsed light, 627.8nm)
- ■ Argon-Ion/Dye laser (continuous wave light, 625-635nm)

The histological appearance of Lewis lung carcinoma 24h after PDT is shown in Fig. 3.3. There was extensive necrosis of the treated tumour but a small number of apparently viable cells remained. The control tumour received no treatment and appeared viable with a small number of necrotic cells.

(e). Detection of the uptake and distribution of HPD by fluorescence

The presence of porphyrin in tumours or other organs was assessed by examining frozen sections for fluorescence. Twenty four hours after i.p. or i.v. injection of HPD, both Lewis lung carcinoma and B16 melanoma showed extensive bright red fluorescence (Fig. 3.4). Weak fluorescence was detected in tumours as soon as 1h after HPD injection, intense fluorescence was detected 2h after injection and had diminished by about half at 24h. Very little fluorescence was observed in tumours 48h after injection of HPD (Table 3.1).

The distribution of porphyrin within the tissues of mice after injecting HPD (50mg/kg i.p.or i.v.) was examined. Red fluorescence was detected in liver, kidney, spleen, skin, heart, lungs but not in the brain. There was no detectable difference in the distribution of fluorescence between mice injected with HPD i.p.or i.v.

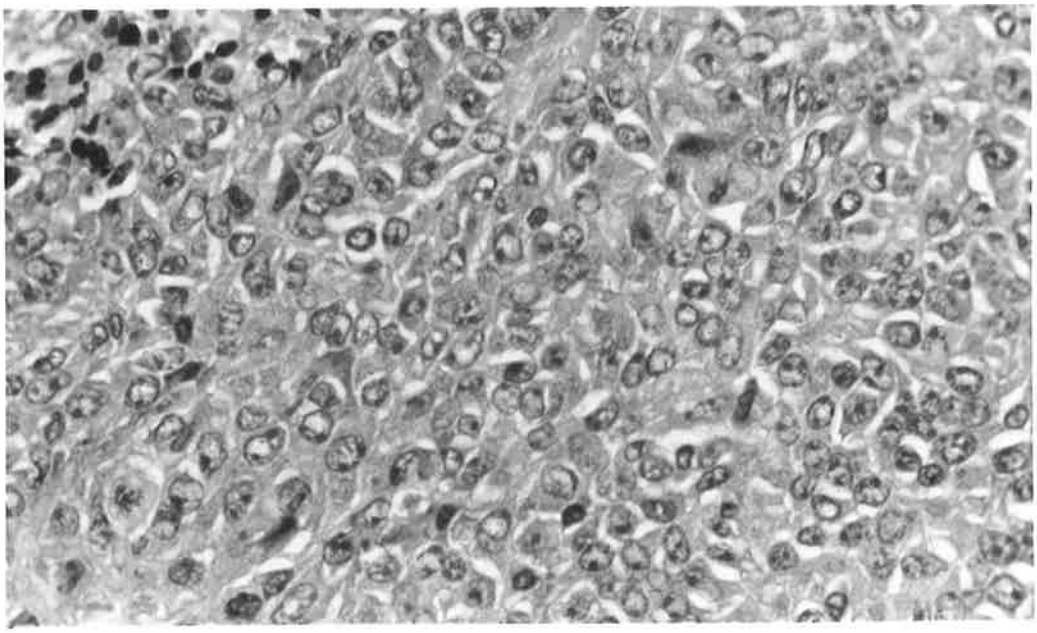
Using the relatively crude detection system available, it was not possible to make any firm conclusions as to the distribution of HPD within a particular tissue. There was more intense fluorescence around the edges of tumours and necrotic areas showed fainter fluorescence.

(f). Measurement of skin photosensitivity by footpad thickness

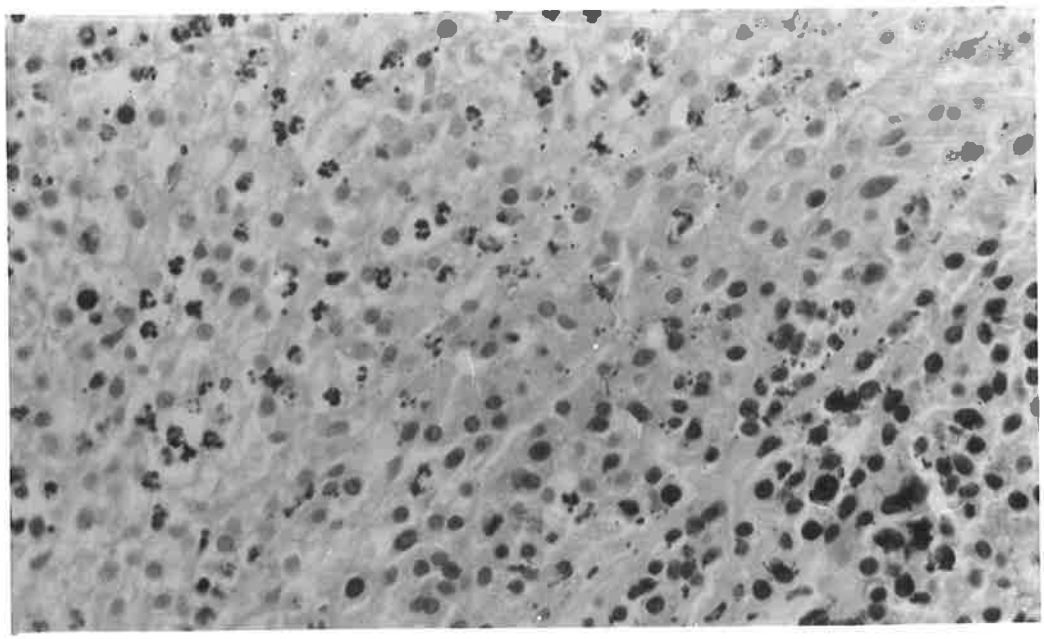
Mice in groups of ten were given HPD (50mg/kg i.p.), the

Fig 3.3

Histological sections of Lewis lung carcinoma



(a). Lewis lung carcinoma before treatment with HPD and light (400x magnification).



(b). Lewis lung carcinoma 24h after treatment with 50mg/kg HPD and 225J/sq cm from the incandescent lamp (400x magnification).

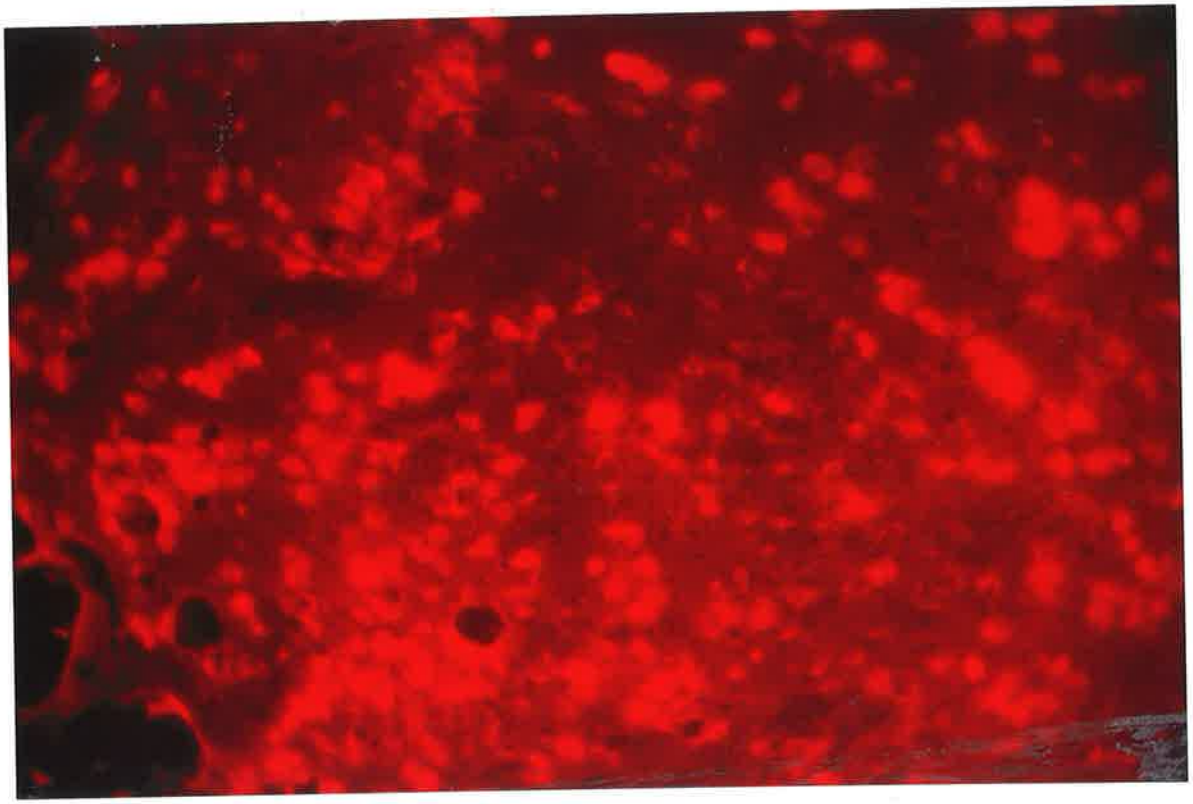


Fig. 3.4

Fluorescence of a frozen section of Lewis lung carcinoma

Tumours were removed 24h after injection of HPD (50mg/kg i.p.) and frozen sections examined under a fluorescence microscope with excitation wavelength 420-490 nm (400x magnification).

Table 3.1

Effect of varying the interval between HPD injection and irradiation

Interval between HPD and irradiation (hours)	TC_{50} (days)	Fluorescence
1	N.D.	Neg
2	8.5±0.5	Strong Pos
24	5.0	Pos
48	3.5±0.5	Weak Pos

N.D.: not done

Mice with Lewis lung carcinoma were injected with HPD (50mg/kg) and irradiated at the intervals shown above. Tumours were removed for fluorescence at the same intervals.

left footpad of the hind leg irradiated, and percentage increase in thickness determined as described above. Before treatment, the ratio between the thickness of the left and right foot (L:R) was 0.99±0.04 and after treatment L:R was 1.34±0.17, a 34% increase. The feet showed considerable erythema and oedema for several days after irradiation which gradually subsided with no permanent damage.

The left feet of mice not given HPD were irradiated with 100sec light. The ratio L:R 24h after irradiation was 1.04±0.003, indicating that light alone had no effect.

(g). Effect of varying the interval between HPD injection and irradiation

The influence of the interval between HPD injection and irradiation on tumour response was examined. Mice with Lewis lung carcinoma were given HPD (50mg/kg i.p) and irradiated with 225J/sq cm from the incandescent lamp, either 2, 24 or 48h after injection. TC_{50} was determined as described above. The size of the tumours at the time of irradiation was the same for all three treatment groups.

The relative efficacies of the treatments are shown in Table 3.1. Two hours after HPD injection, there was a very strong tumour response which gradually diminished over the next 48h. Efficacy of tumour destruction correlated with the relative intensity of fluorescence in the tumours (section (e)). The effect of irradiating 1h after HPD injection was not examined as there was very little fluorescence detected in the tumour. An interval of 24h between HPD injection and irradiation was chosen for most studies as it most closely approximated the clinical situation.

(h). Treatment of B16 melanoma by PDT

Mice were transplanted with B16 melanoma as described in section (b). After 7 to 10 days, tumours were 5-7mm in diameter and mice in groups of 10 were injected with HPD (30mg/kg i.p.). Twenty four hours later, mice were irradiated as described above with 100-300 sec (110-340J/sq cm) red light from the incandescent lamp. TC_{50} was determined as described above.

B16 melanoma was treated more effectively with PDT than Lewis lung carcinoma. Only half the light dose and a lower HPD dose were required to obtain an equivalent tumour response. TC_{50} was proportional to the light dose (Fig. 3.5), confirming the validity of TC_{50} as a measure of the efficacy of treatment. Histological examination of B16 melanoma 24h after PDT showed extensive necrosis with a few viable tumour cells remaining.

(i). Discussion

The assay described above was designed to quantify the photocytotoxic activity of HPD in vivo. Mice were transplanted subcutaneously with Lewis lung carcinoma or B16 melanoma and treated when the tumours were 5-7mm diameter. Twenty four hours after a standard treatment schedule of 50mg/kg HPD and 225J/sq cm light for Lewis lung carcinoma and 30mg/kg HPD and 110J/sq cm for B16 melanoma, 9 to 10 out of 10 tumours were no longer palpable. The measure of efficacy of the treatment was the time for 5 out of 10 tumours to recur, called TC_{50}.

Provided uniformly sized tumours were used, the reproducibility of TC_{50} between assays was excellent. With Lewis lung carcinoma, HPD (50mg/kg) and light (225J/sq cm) resulted in a TC_{50} of 4.6 ± 0.6 days from nine separate assays. These conditions have been used to test all new batches of HPD.

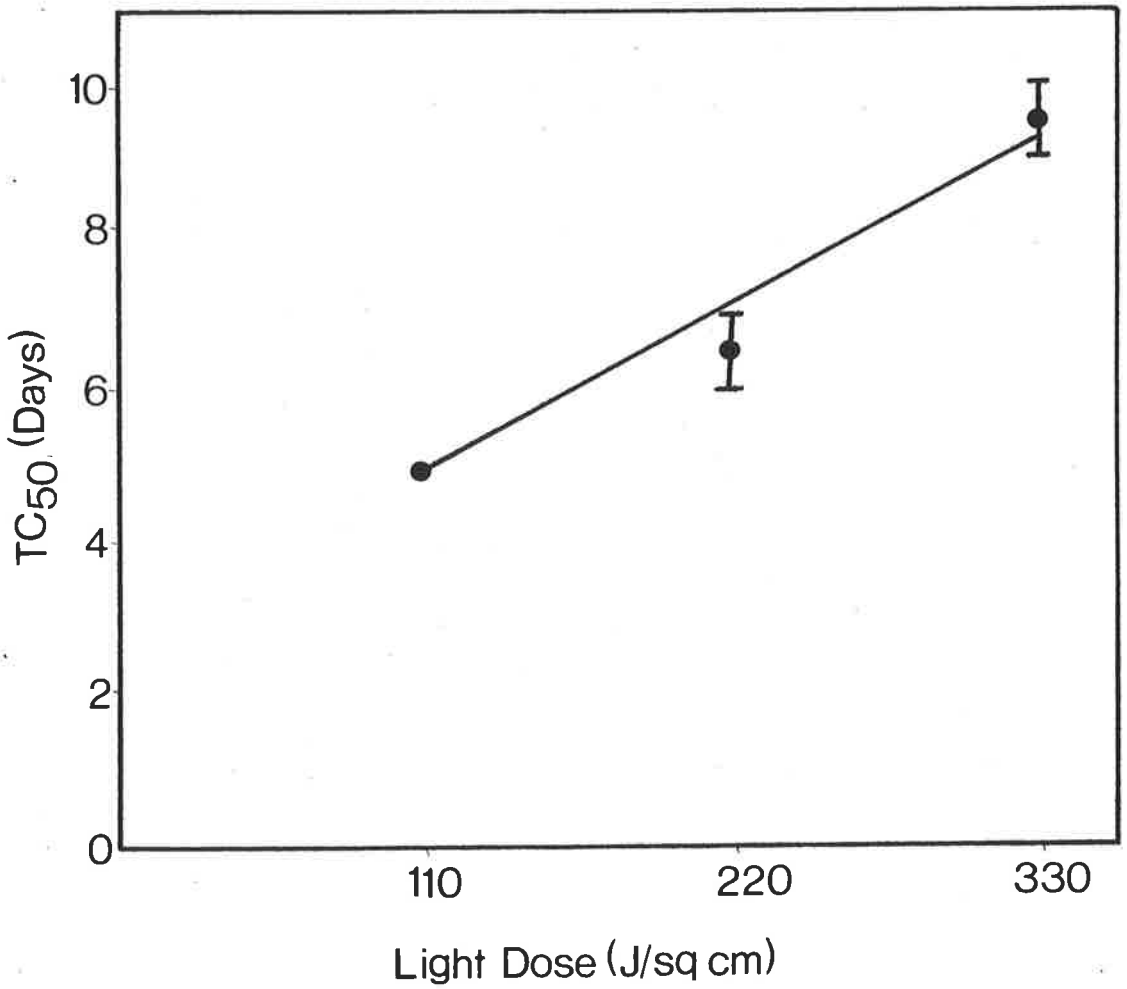


Fig. 3.5

Relationship between TC_{50} and light dose for B16 melanoma

Mice were given HPD (30mg/kg i.p.) and irradiated 24h later.

For all experiments, at least 2 groups of ten mice were tested and the mean and standard deviation of the TC_{50} calculated.

There was a linear relationship between doses of HPD or light and TC_{50} (Figs. 3.1 and 3.2). A longer TC_{50} implies slower recurrence, hence greater initial destruction of tumours. Repopulation studies by Stephens and Steel [1980] cast doubt on this assumption. They found growth delay of Lewis lung carcinoma was not linearly related to the initial log survival after radiotherapy, measured by colony formation. This implied the rate of regrowth of the tumour was not directly related to the degree of initial cell kill. In the present situation, TC_{50} appears a valid measure of the effectiveness of the treatment as more stringent treatment conditions gave longer control of the tumours.

A relatively high dose of HPD (25-60mg/kg) was required to obtain a satisfactory tumour response. B16 melanoma was more sensitive to PDT, since lower HPD and light doses were adequate to achieve the same response as Lewis lung carcinoma. Both the HPD and light doses required for adequate treatment of Lewis lung carcinoma were in good agreement with Ellingsen et al. [1984]. They obtained good destruction of tumours with 50mg/kg HPD and irradiating 24h later with 160-360J red light. Other studies using different strains of mice or different tumour lines have used 3.5-10mg/kg HPD [Dougherty et al., 1983; Patrice et al., 1983]. The higher HPD dose necessary to obtain an adequate response in Lewis lung carcinoma may reflect inadequate light penetration due to the pigmentation of the tumour and the black skin of the mouse. The tumour may also be relatively resistant to the treatment. However the requirement

for a high dose of HPD does not affect the validity of the assay as a measure of relative efficacy of the treatment.

Uptake of HPD into tumours and other tissues was assessed by fluorescence of frozen sections. The distribution of HPD fluorescence within the tumours was largely peripheral. This agrees with Lipson et al. [1961a] and Carpenter et al. [1977] who reported more intense fluorescence around the edges where the tumour invaded normal tissue, and around blood vessels. Bright fluorescence was observed in all tissues except brain. Relative concentrations of HPD in the tissues could not be determined accurately by the methods available. Distribution of HPD in the body has been discussed in Chapter 1. The above results are in agreement with the reported widespread distribution of HPD rather than specificity of uptake in tumours.

Intravenous administration of HPD resulted in slightly better tumour destruction than intraperitoneal injection of HPD but there was no detectable difference in the intensity of fluorescence in the tumours. Carpenter et al. [1977] reported brighter fluorescence and greater tumour selectivity with i.v injection of HPD in mice with transplanted mammary adenocarcinoma. This discrepancy probably reflects the poor sensitivity of our techniques for detecting fluorescence. Tomio et al. [1980] reported i.v. or i.p. injection of HP resulted in equivalent degrees of tumour necrosis in Yoshida ascites hepatomas in rats. However they were using a commercial HP preparation which always contain photosensitizing contaminants (HVD, PP and aggregated porphyrins). Considerable variation in the photoactivity of HP has been reported by different workers [Dougherty, 1983] and our HP preparations were not photoactive.

This will be discussed further in Chapters 4 and 5.

Mice were anaesthetised with sodium pentobarbitone (60mg/kg) for ease of irradiation. There may be some interactions between PDT and the anaesthetic. Peacock and Stephens [1978] reported that the response of B16 melanoma to melphalan therapy was inhibited by Saffan, a steroid anaesthetic, but not by sodium pentobarbitone. Gomer and Razum [1984] showed that the degree of skin damage after PDT was not affected by sodium pentobarbitone anaesthesia. Anaesthetising the mice with sodium pentobarbitone would be be a standard factor during all PDT treatments.

No difference was detected in the efficacy of either pulsed or continuous wave laser light in causing destruction of Lewis lung carcinoma in HPD-sensitized mice [Cowled et al., 1984]. Pulsed light is as least as effective as continuous wave laser light and some studies suggest it may be more efficient in PDT. Fluorescence decay studies indicated HPD radicals were formed when HPD was irradiated with pulsed but not continuous wave light [Andreoni et al., 1982b]. They observed increased killing of rat epithelial cells by pulsed light compared to continuous wave light. The difference between their results and the above may be due to their use of lower wavelength (337.1nm), slower pulses of 30Hz or the different methods of preparing the HPD. Hisazumi et al. [1985] reported pulsed laser light from a gold vapour laser was twice as effective in causing photodynamic destruction of mouse tumours. The discrepancy with the above results may be due to poor sensitivity of our assay.

CHAPTER 4

IN VITRO PHOTOCYTOTOXICITY OF SOME OF THE COMPONENTS OF HPD

(a). Introduction

Haematoporphyrin derivative (HPD) was first described by Lipson [Lipson and Baldes, 1960a; Lipson et al., 1961a] and claimed to be superior to HP as a tumour localizer. HPD is a complex mixture of porphyrins and since these initial papers, the chemical composition of HPD and also the nature of the porphyrin(s) responsible for the tumour localizing and photocytotoxic activity have been studied extensively.

HPD is prepared from commercial HP in two steps [Lipson et al., 1961a; Dougherty et al., 1978]. HP is first acetylated by acetic acid/sulphuric acid and the product (HPD-solid) is obtained by precipitation at pH6.0. HPD-solid is then dissolved in 0.1N sodium hydroxide and stood at room temperature for 1h before being neutralized with 0.1N hydrochloric acid. This is the solution used clinically and will be referred to as HPD.

In early reports [Dougherty et al., 1978], HPD was separated into at least 4 bands by thin layer chromatography but no chemical identification was attempted. Methods have been recently established to separate and analyse porphyrins by reverse phase high performance liquid chromatography (HPLC). Clezy et al. [1980] analysed the esters of HPD-solid and showed HP diacetate was the main porphyrin component. HPLC analysis of HPD-solid by Bonnett et al. [1981] showed the main components were the O-acetyl and O,O-diacetyl derivatives of HP. Cadby et al. [1982; 1983] confirmed that HPD-solid was composed of HP, HP diacetate, the isomers of HP hydroxy acetate

and HP vinyl acetate and a small amount of protoporphyrin (PP). HPD (clinical solution) consisted of 45-50% HP, smaller amounts of hydroxyethyl vinyl deuteroporphyrin (HVD) and PP with a considerable amount of an unidentified hydrophobic component. Moan and coworkers have also analysed HPD by HPLC and reported 4 main components, HP, the two isomers of HVD and an unidentified hydrophobic component [Moan and Sommer, 1981; Moan et al., 1982b; 1982c; 1983b]. Kessel [1982b] also confirmed that the main components of HPD were HP, HVD, PP and another hydrophobic component.

The chemical composition of HPD is further complicated by the fact that porphyrins self-aggregate in solution. Dougherty et al. [1983] showed three main components in HPD on a Biogel p10 polyacrylamide gel column. The fastest-running band eluted in the void volume, which indicated it had a molecular weight of over 20,000. Ultracentrifugation studies [Swincer et al., 1985] showed the fastest-running band from a Biogel p10 column had an average molecular weight of 21,500. The intermediate band was 8,000 and the slowest running band was 6,500. Fluorescence decay data also provided evidence for the presence of stable aggregates in HPD or commercial HP [Andreoni et al., 1982a; Smith, 1985].

Determining the nature of the tumour-localizing and phototoxic component in HPD is important in developing more effective therapies. A pure compound of known structure could be used. Removal of inactive porphyrins may increase the specificity of uptake into tumours with a reduction in skin photosensitivity. A lower total dose of porphyrin could also be used. Understanding the nature of the active porphyrin could

also help in designing other more efficient tumour photosensitizers. In this context, porphyrin C has been investigated by Scourides et al. [1985]. It is a porphyrin with a very short retention time in the body and by virtue of this, will reduce long term photosensitivity.

The photoactivity in vitro of some of the components of HPD and also of porphyrin C will be examined in the following studies.

(b). Preparation and analysis of some of the components of HPD

(i). Biogel p10 chromatography

Method

Biogel p10 polyacrylamide beads (Biorad Laboratories) were allowed to swell for 24h in degassed water buffered to pH7.0 with trace amounts of phosphoric acid and triethylamine. Columns (15.0X1.0cm) were poured and equilibrated with pH7.0 water. HPD (100ul of 5mg/ml) was loaded and the column eluted with pH7.0 water. The column flow rate was 0.5ml/min. One min fractions were collected, diluted with 3ml of 1:1 ethanol:0.1M NaOH and optical density was read at 397nm. For phototoxicity assays, the porphyrin peaks were detected by eye and pooled.

Results

A typical chromatogram of HPD is shown in Fig. 4.1. Three main peaks were detected. A dark brown peak eluted in the void volume of the column (5ml) and overlapped with a second dark red peak. The broad slowest-running peak consisted of pink material. The three peaks have been designated HPD aggregate, HPD intermediate and HPD slow fractions respectively.

It was not possible to obtain these three peaks consistently. Occasionally, only the aggregate and slow

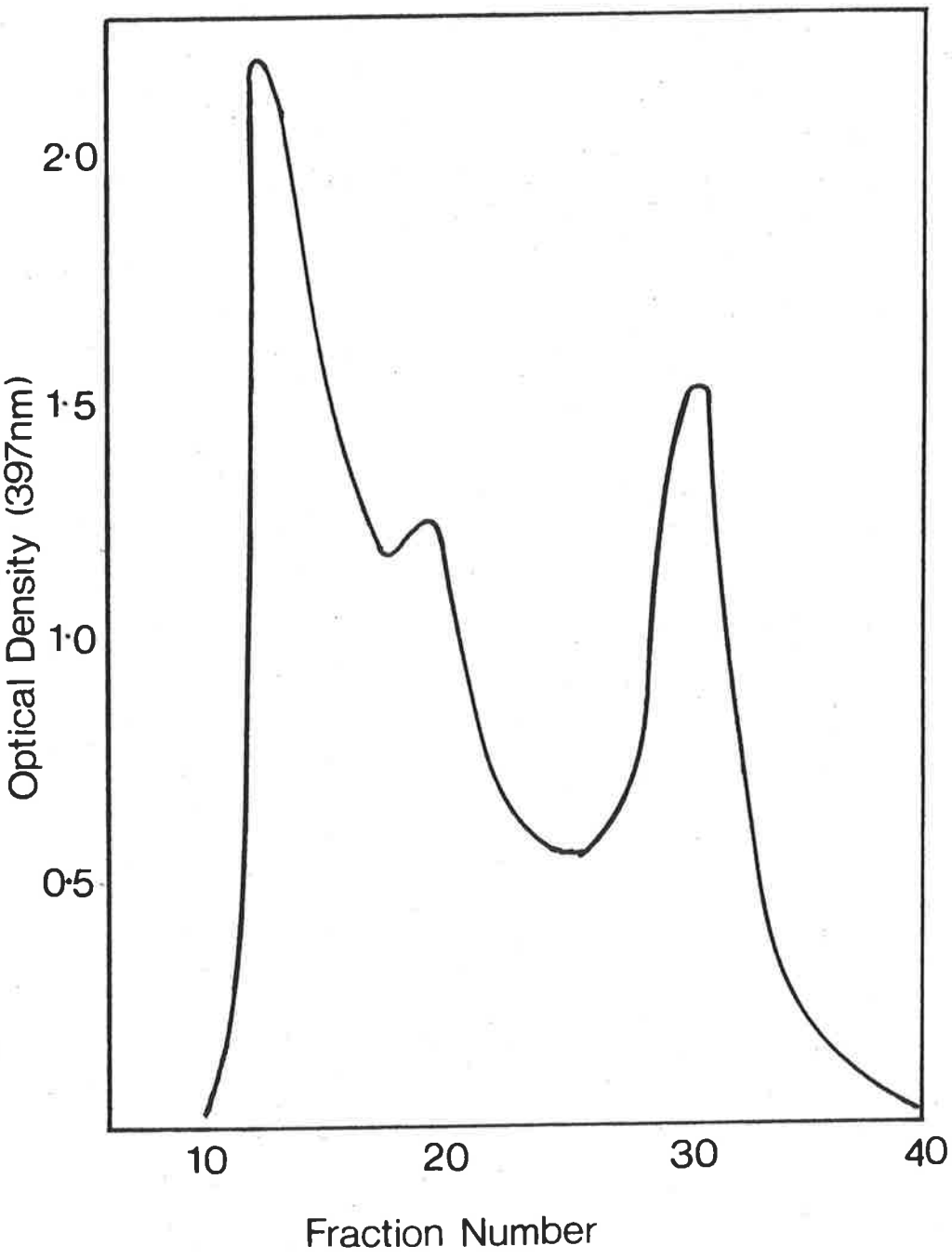


Fig 4.1

Separation of HPD by Biogel p10 chromatography

HPD (100ul of 5mg/ml) was loaded onto the column and eluted with water buffered to pH7.0 with phosphoric acid and triethylamine.

fractions were detected (Fig. 4.2). The reasons for this discrepancy were not clear but if freshly distilled water buffered to pH7.0 and degassed was used, the three bands could be obtained reproducibly.

(ii). Preparation of pure porphyrins

Pure HP and HVD were prepared by Dr A.G. Swincer, Organic Chemistry Department, The University of Adelaide, by acid-ether extraction of HPD by a published procedure [Cowled et al., 1985]. HVD was separated on a Biogel p10 column into three aggregation states as described above. PP was prepared by Dr. A.G. Swincer from HP by a procedure described in the literature [Dinello and Chang, 1978]. PP ran as a single fast-running peak on Biogel p10 columns, indicating that it was highly aggregated. All pure porphyrins were analysed by HPLC before testing for photoactivity.

Porphyrin C solid was prepared by Dr. P.A. Scourides, Ludwig Institute for Cancer Research, Royal Melbourne Hospital [Scourides et al., 1985] and dissolved in 0.1N NaOH. The pH was adjusted to 7.4 with 0.1N HCl and water and solid NaCl added to give an isotonic solution with a final concentration of 5mg/ml. All solutions were degassed and bubbled with N_2. Solutions were manipulated and stored under a nitrogen atmosphere. $O.D._{397}$ of porphyrin C was measured after diluting in 1:1 ethanol:0.1N NaOH.

(iii). High performance liquid chromatography

HPLC was performed using a Waters Novapak C18 cartridge. The column was equilibrated with 2.5mM tetra-n-butylammonium phosphate (Unichrom) in 85% methanol (pH3.0, buffered with phosphoric acid and triethylamine). Porphyrin samples were injected in this solvent and the column eluted with aqueous

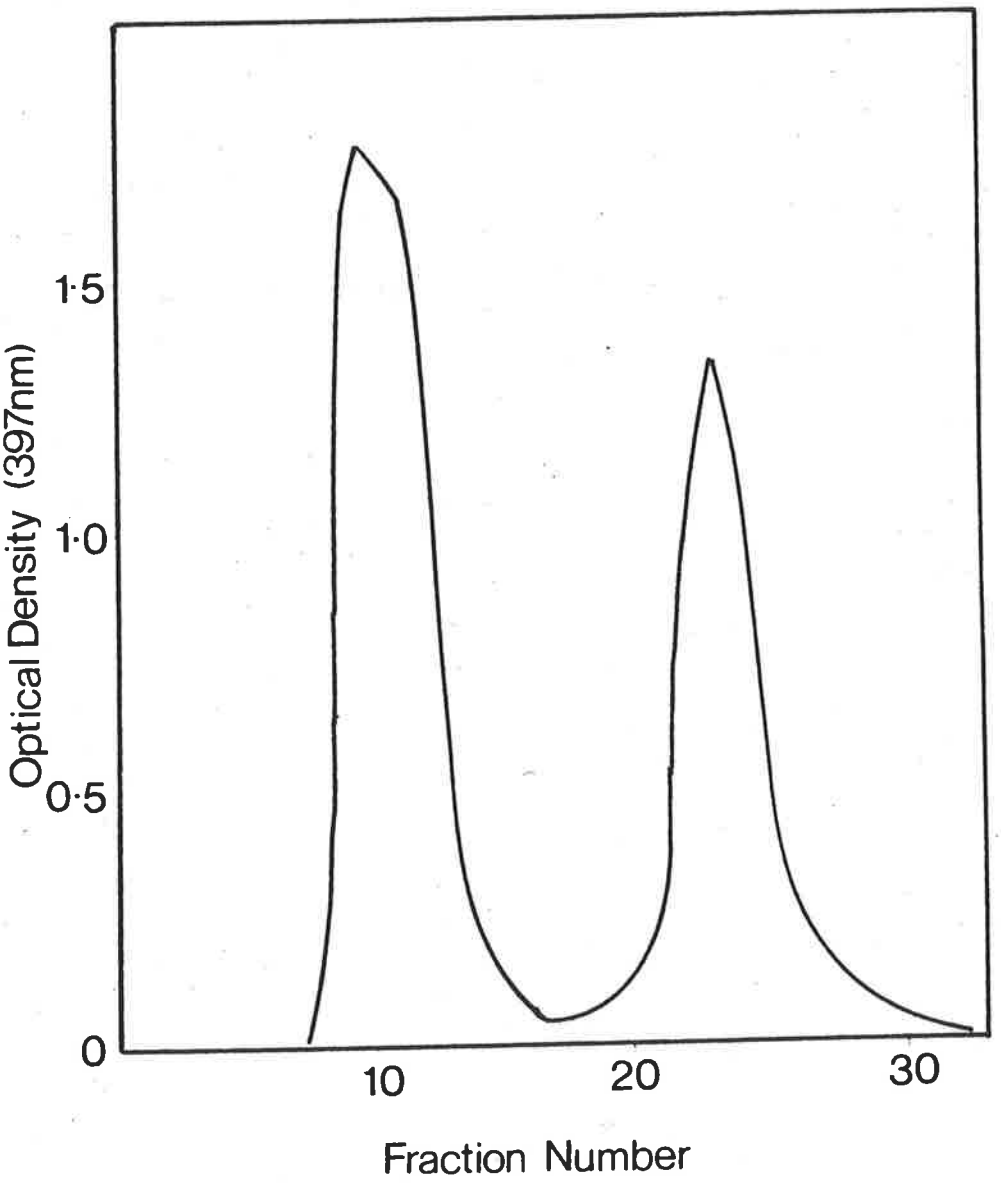


Fig. 4.2

Separation of HPD by Biogel p10 chromatography

HPD (100ul of 5mg/ml) was loaded onto the column and eluted with water buffered to pH7.0 with trace amounts of phosphoric acid and sodium hydroxide.

methanol (1:9, pH7.2). The detector was set at 397 nm. Under these conditions all of the injected material was eluted from the column. The HPLC analysis of HPD is shown in Fig. 4.3 and was in agreement with previously published reports [Cadby et al., 1982; 1983; Moan et al., 1982b; 1982c; 1983b]. The preparations of the individual porphyrins were of high purity (Fig. 4.4). HPLC analysis of the HPD fractions prepared by Biogel p10 chromatography shows the aggregate fraction had the greatest proportion of poorly-resolved hydrophobic porphyrins and the slow fraction consisted largely of HP and HVD which are the most hydrophilic porphyrins in HPD (Fig. 4.5).

(iv). Ultrafiltration

Amicon YM10 ultrafilters with a molecular weight cutoff of 10,000 were used for the preparation of aggregated and less aggregated fractions of HPD. Twenty ml of HPD was loaded onto the filter and the first 10ml passed through the filter was collected. This was called HPD passed fraction. Biogel p10 chromatography indicated this fraction had a relatively low molecular weight, since it comigrated with HPD slow fraction. The porphyrin retained by the ultrafilter was washed with 150ml of saline. The final 10ml retained by the filter was called HPD retained fraction. Biogel p10 chromatography showed this fraction consisted mostly of fast-running aggregates with a small contamination by the slow fraction.

(v). Polyacrylamide gel electrophoresis

Method

Electrophoresis was carried out using the flat bed LKB Multiphor apparatus in a tris-glycine pH 8.9 system. The buffer stock solution was prepared by dissolving 75.1g glycine and

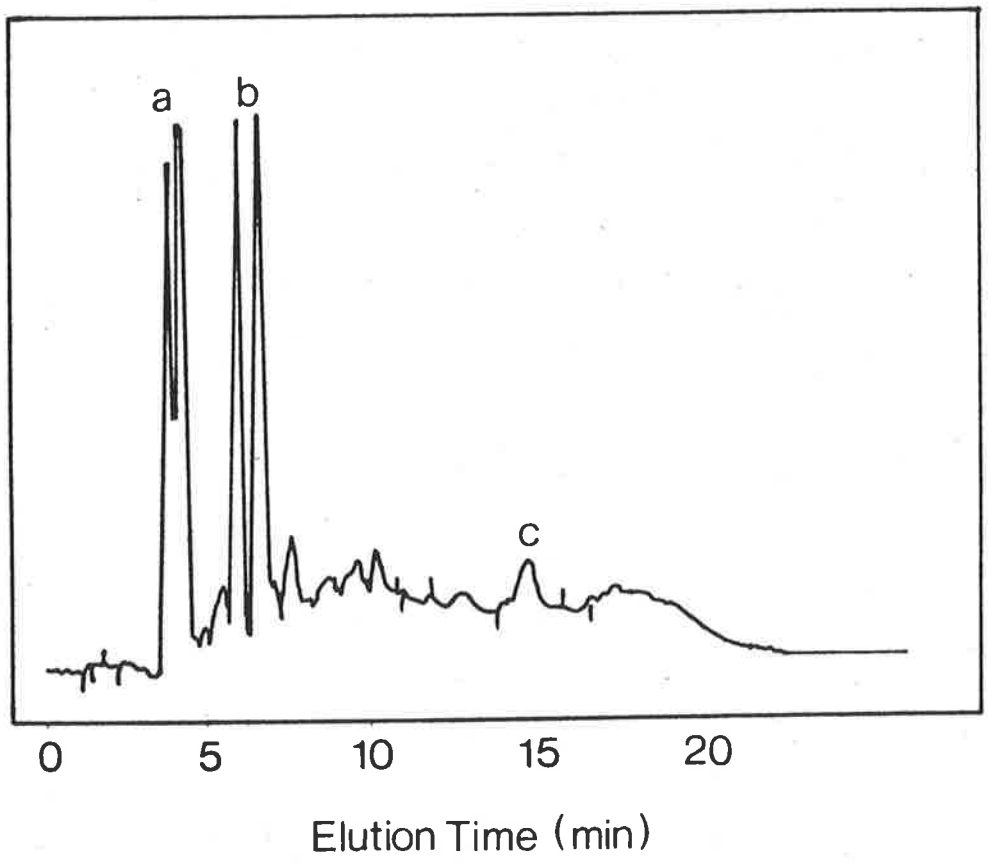


Fig. 4.3

HPLC analysis of HPD

The column was run under standard conditions described in the text.

a: HP

b: HVD

c: PP

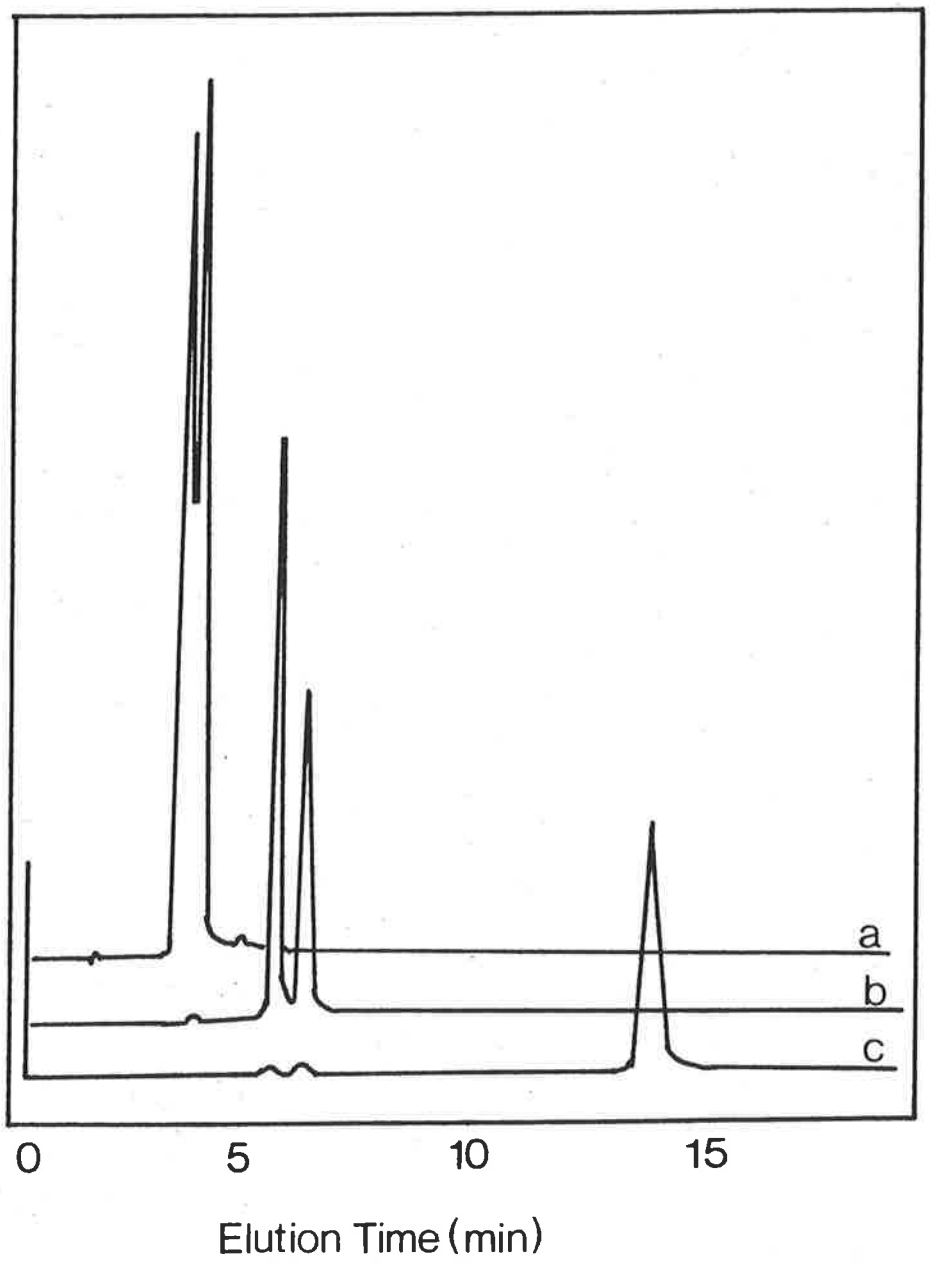


Fig. 4.4

HPLC analysis of pure porphyrins

The column was run under standard conditions described in the text.

a: HP

b: HVD

c: PP

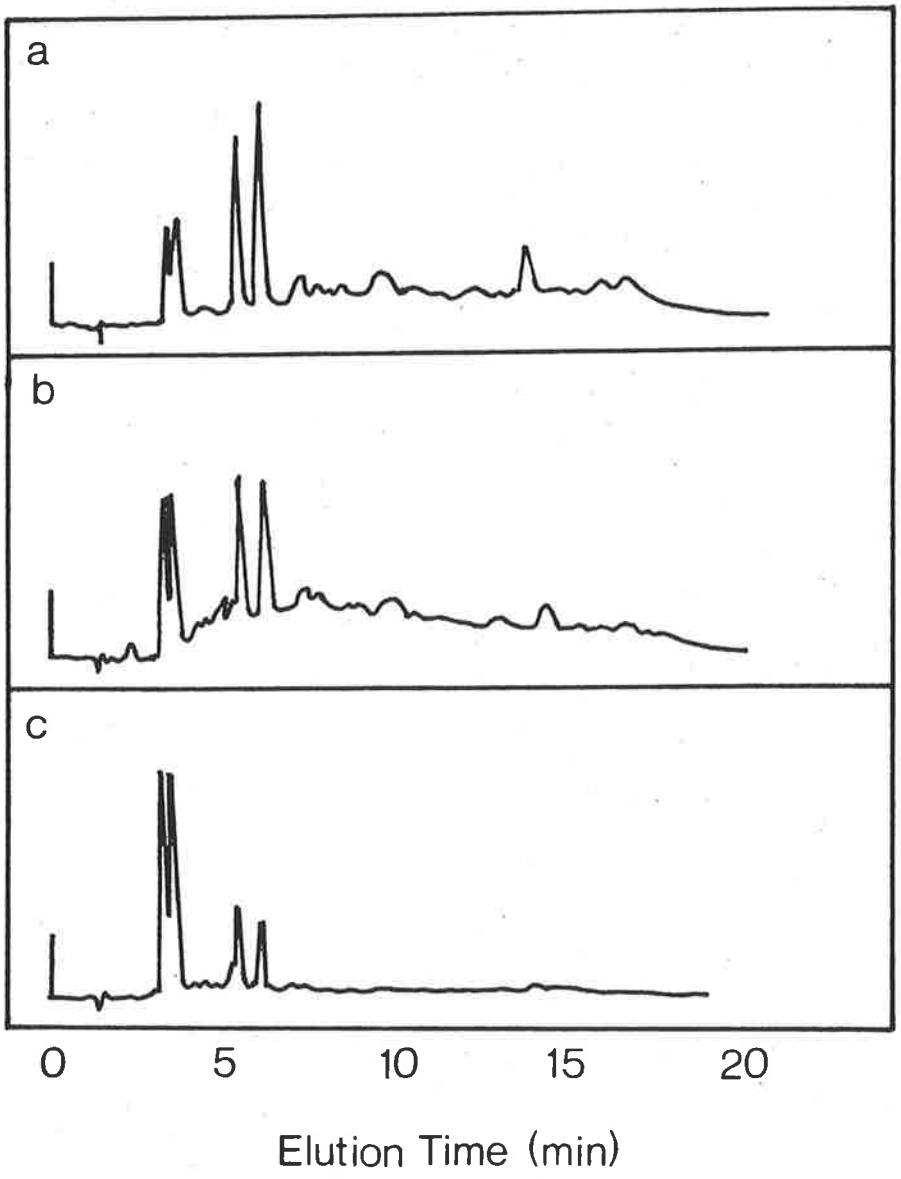


Fig. 4.5

HPLC analysis of HPD fractions isolated by Biogel p10 chromatography

The column was run under standard conditions described in the text.

a: HPD aggregate (Fast-running fraction)

b: HPD intermediate fraction

c: HPD slow fraction

2.5g sodium azide in 5 litres of distilled water and adjusting the pH to 8.9 with solid Trizma base (Tris(hydroxymethyl) aminomethane, Sigma). Electrode buffer was prepared by mixing 1 part buffer stock with 1 part distilled water.

Gels were prepared using the recipes in Table 4.1. Some gels were poured between plain glass plates and 20ul wells cut in the gel. Alternatively, a 10ul slot former (LKB) was used. Gels were stored overnight at 4^{o} in a humidified chamber before use.

The plates were pre-electrophoresed for 30min at 50mA before use. HPD (10 or 20ul of a 5mg/ml solution) was applied to the plates and concentrated for 10min at 20mA. The field strength was then increased to 15volts/cm for approximately 3h. The plates were examined under a ultraviolet light and photographed. The porphyrin bands were cut out of the gel and soaked in PBS for 48 hours in the dark. The material recovered was analysed by HPLC and tested for photocytotoxicity in vitro. HPD retained fraction and HPD passed fraction (YM10 ultrafilter) were also analysed by electrophoresis as above.

Results

Electrophoresis of HPD resulted in its separation into two bands (Fig. 4.6), a fast-moving brown band and a slower moving pink band. Separation between the bands was cleanest with 3.5% and 5% gels. With a 7.5% gel, the brown band was considerably streaked back towards the pink band. The preparative runs were carried out using 5% gels. HPD retained fraction ran as a faster-moving brown band with a small contaminant of the slower-moving pink band. HPD passed fraction consisted entirely of the slow-moving pink band. Under ultraviolet light, the pink band showed intense salmon pink fluorescence while the brown

Table 4.1.

Composition of polyacrylamide electrophoresis gels

Solution	3.5% Gel	5.0% Gel	7.5% Gel
Distilled water	19.3	14.9	7.5
Tris-glycine stock buffer (pH 8.9)	33.0	33.0	33.0
Acrylamide solution*	10.4	14.8	22.2
Ammonium persulphate (15 mg/ml)	3.2	3.2	3.2
TEMED	0.1	0.1	0.1
Total volume (ml)	66.0	66.0	66.0

* Acrylamide solution: Dissolve 22.2g acrylamide (BDH) and 0.6g Bis acrylamide in 100 ml distilled water and filter through a Whatman no. 1 filter.

Fig. 4.6

Separation of HPD by polyacrylamide gel electrophoresis

The conditions are described in the text.

a: HPD

b: HPD aggregate (retained by Amicon YM10 ultrafilter)

c: HPD passed fraction (passed by Amicon YM10 ultrafilter)

band was not fluorescent.

HPLC analysis of the two gel extracts (Fig. 4.7) indicated that the fast and slow migrating bands were of similar composition to HPD aggregate and HPD slow fraction isolated from a Biogel p10 column. The two gel extracts were tested for photocytotoxicity *in vitro* and the results given below (Fig. 4.11).

(c). Photocytotoxicity of some of the components of HPD

(i). Method

Photocytotoxicity *in vitro* was tested using the assay described in Chapter 2. Porphyrin solutions were prepared in saline and added to ^{51}Cr-labelled Raji cells so that the final optical density at 397nm was 4.0. After incubating for 1h at 37^{o}, the cells were washed in RPMI 1640 and resuspended in PBS ($0.5X10^{6}$/ml). In some experiments, ^{51}Cr-labelled cells were resuspended in PBS ($0.5X10^{6}$/ml), porphyrins were added and the cells irradiated at once. Alternatively, cells were incubated in porphyrin for 2lh, then resuspended in RPMI 1640/10%FCS and incubated for 4h to allow efflux of porphyrins from the cells.

The photoactivity of each porphyrin was assessed in several separate experiments using fresh preparations of porphyrin. Before testing, each porphyrin was checked for purity by HPLC and its aggregation state was checked by Biogel p10 chromatography. HPD was run as a standard in all assays.

Uptake of porphyrin after 1h incubation ($O.D._{397}=4.0$) was assessed by fluorescence microscopy as described in Chapter 2.

(ii). Photocytotoxicity of porphyrins after 1h Incubation

The photoactivity of HPD and HPD fractions prepared by Amicon YM10 ultrafiltration was compared (Fig. 4.8). HPD and

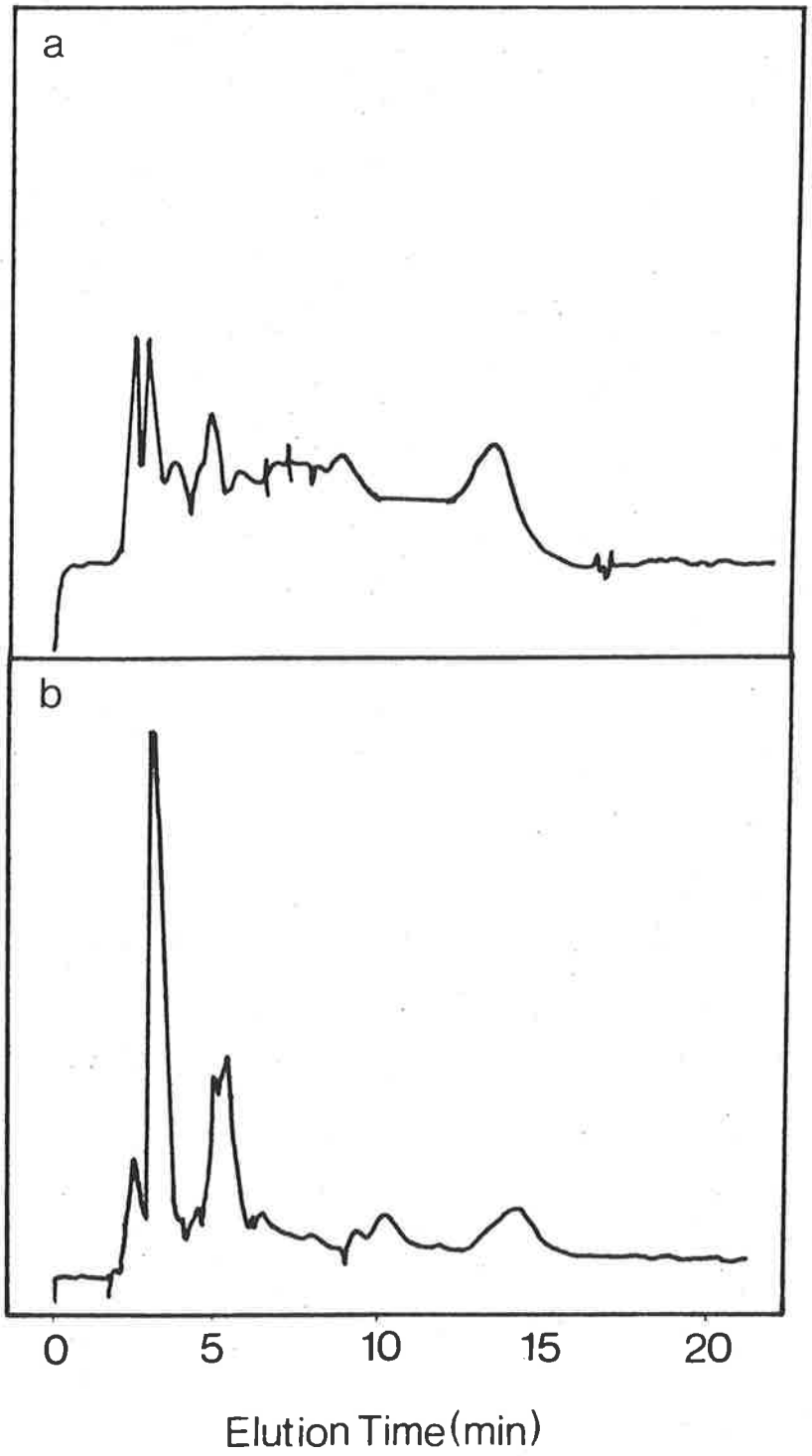


Fig. 4.7

HPLC analysis of porphyrin fractions prepared by polyacrylamide gel electrophoresis

The column was run under standard conditions described in the text.

a: Fast-running brown fraction.

b: Slow-running pink fraction.

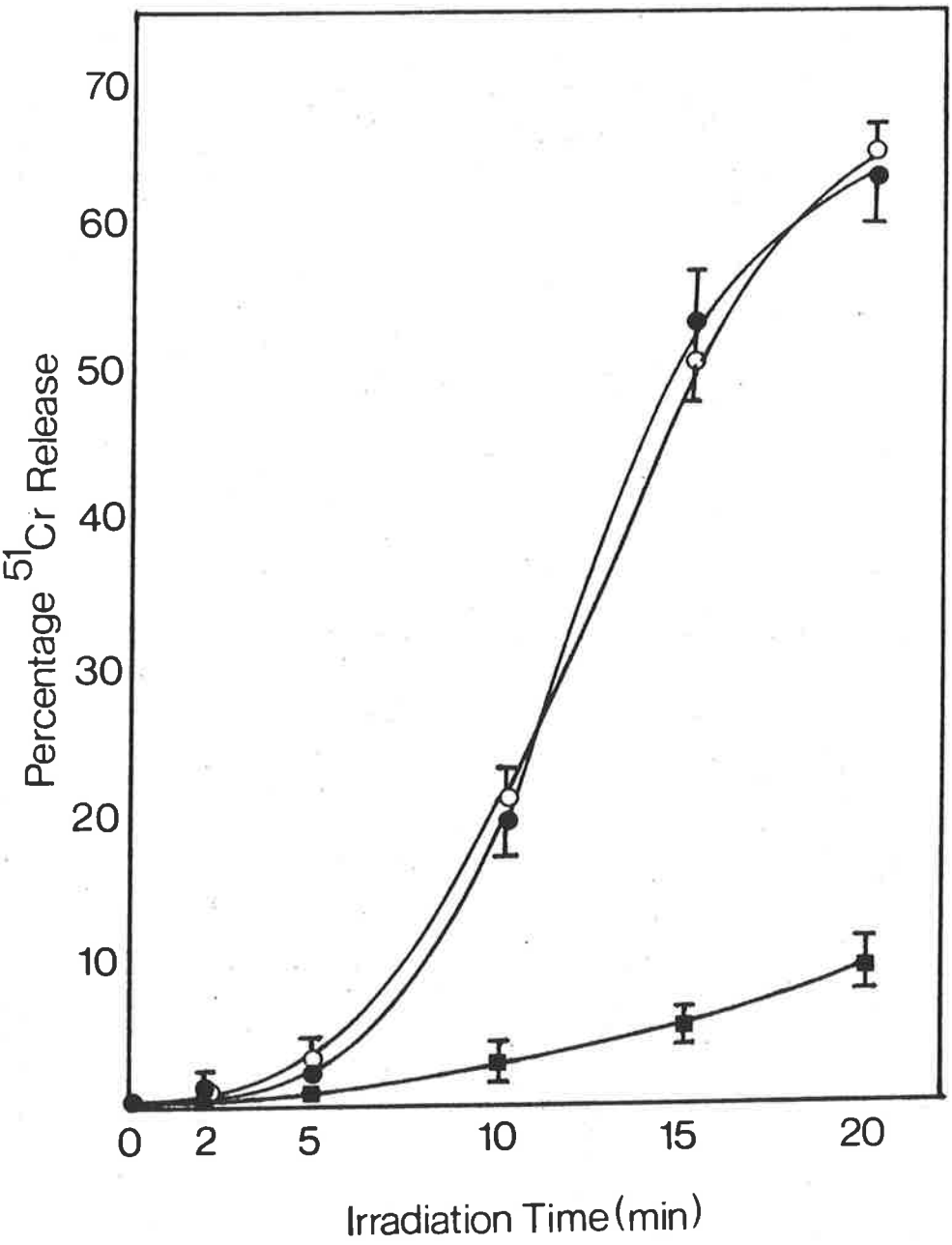


Fig. 4.8

Photoactivity of porphyrin fractions prepared by Amicon YM10 ultrafiltration

^{51}Cr-labelled Raji cells were incubated for 1h with porphyrins (O.D.$_{397}$=4.0) and irradiated.

- ● HPD
- ○ HPD retained fraction
- ■ HPD passed fraction

HPD retained fraction resulted in identical profiles of ^{51}Cr release but the material passed by the YM10 filter was nearly inactive.

The three fractions isolated by Biogel p10 chromatography were compared (Fig. 4.9). Incubating the cells in the fast-moving aggregate fraction resulted in the fastest ^{51}Cr release (similar to HPD), the intermediate fraction showed intermediate activity and the slowest moving fraction, the slowest ^{51}Cr release.

The photoactivity of the pure porphyrins is shown in Fig. 4.10. PP was the most active and HP was inactive. Of the three aggregation states of HVD, the aggregate was the most active, the intermediate fraction the next active and the non-aggregate fraction least active. The photoactivity of HPD was similar to HVD aggregate.

The relative photoactivities of the two fractions prepared by polyacrylamide gel electrophoresis was examined. The faster-moving brown fraction was more photoactive than the slower pink band (Fig. 4.11). This behaviour was consistent with the two bands having a similar composition to the aggregate and slow fractions from a Biogel p10 column.

(iii). Correlation between photocytotoxicity and fluorescence uptake of porphyrins

Uptake of porphyrin into Raji cells resulted in bright red fluorescence (Fig. 4.12). Fluorescence was detected after cells were incubated in HPD, HVD, PP, HPD aggregate, HPD retained fraction and HPD intermediate fraction (Table 4.2). Cells incubated in HPD passed fraction (YM10 ultrafilter) and HPD slow fraction (p10 column) were weakly positive and cells

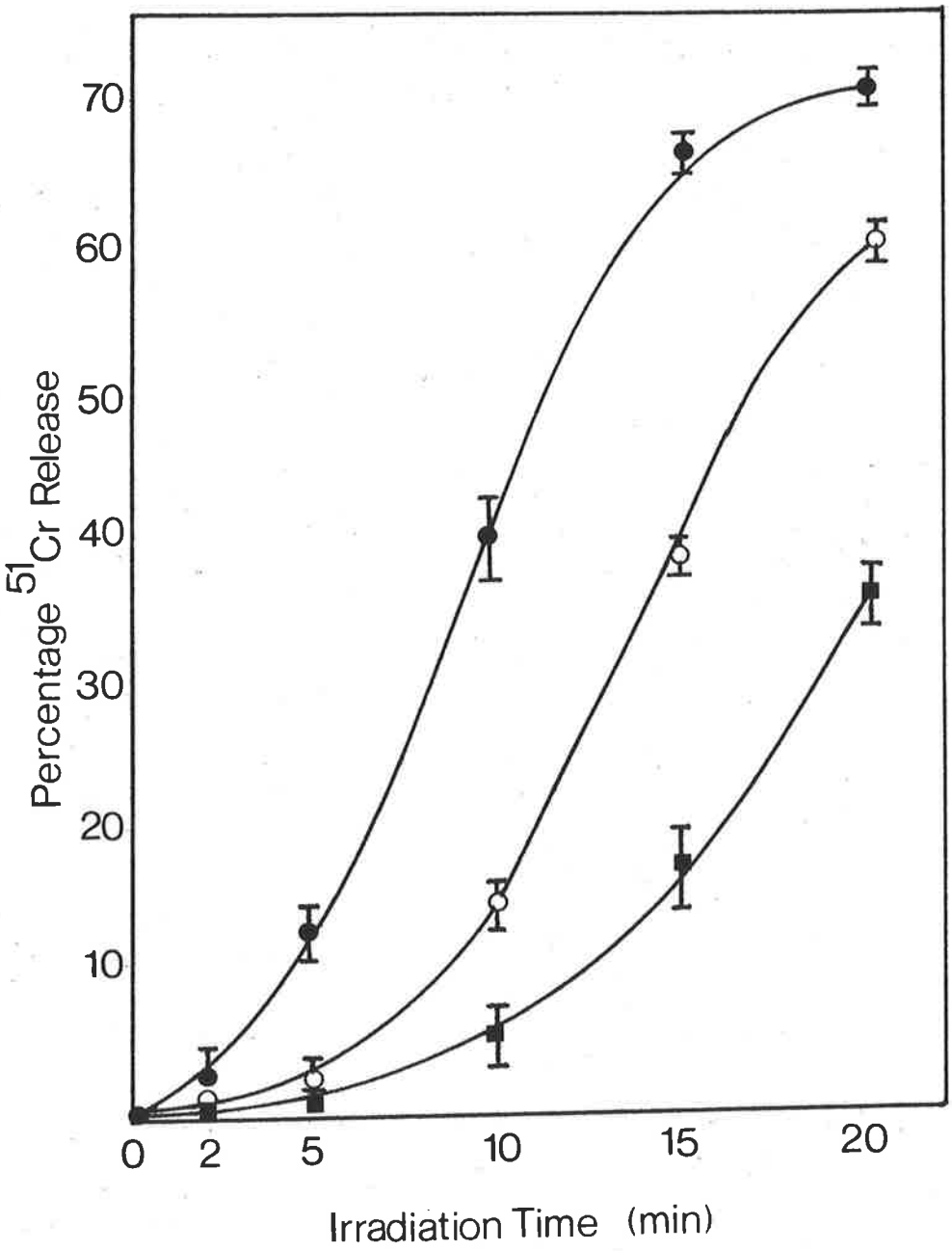


Fig. 4.9

Photoactivity of porphyrin fractions isolated by Biogel p10 polyacrylamide gel chromatography

^{51}Cr-labelled Raji cells were incubated for 1h with porphyrins (O.D.$_{397}$=4.0) and irradiated.

● HPD aggregate fraction

○ HPD intermediate fraction

■ HPD slow fraction

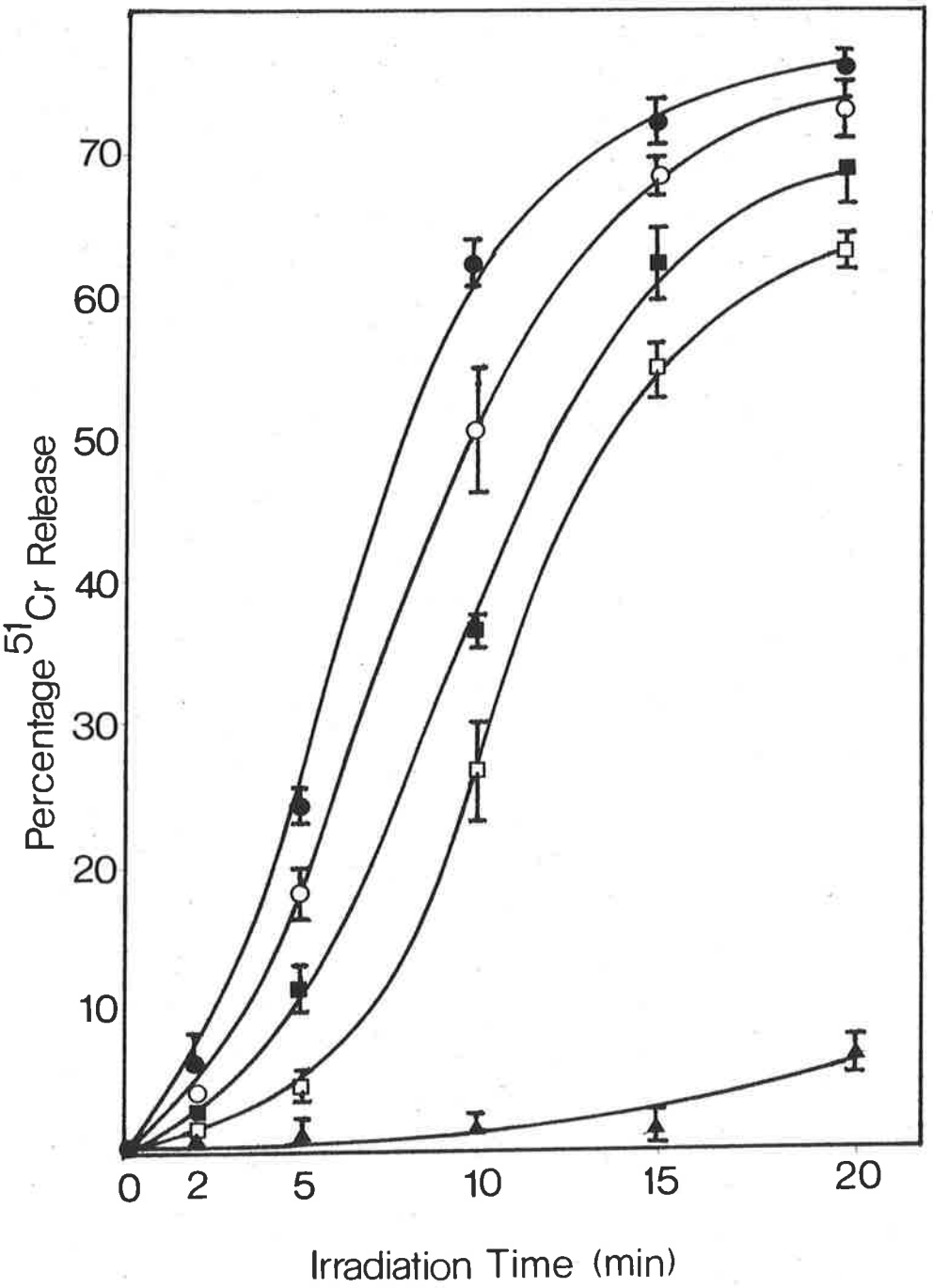


Fig. 4.10

Photoactivity of pure porphyrins

^{51}Cr-labelled Raji cells were incubated for 1h with porphyrins (O.D.$_{397}$=4.0) and irradiated.

- ● PP
- ○ HVD aggregate (p10 column)
- ■ HVD intermediate fraction (p10 column)
- □ HVD slow fraction (p10 column)
- ▲ HP

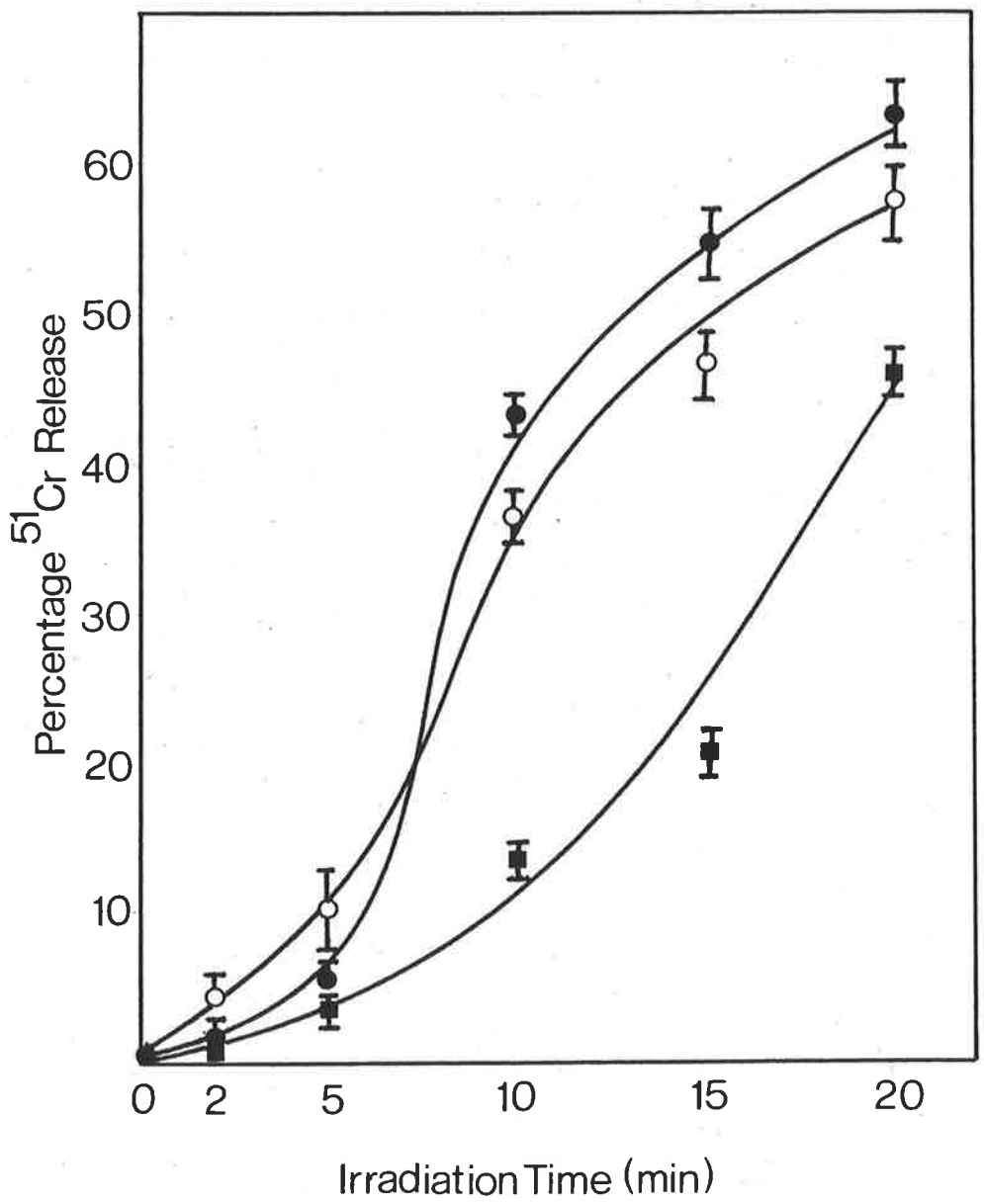


Fig. 4.11

Photoactivity of porphyrin fractions prepared by polyacrylamide gel electrophoresis

^{51}Cr-labelled Raji cells were incubated for 1h with porphyrins (O.D.$_{397}$=4.0) and irradiated.

● HPD

○ HPD fast-running band

■ HPD slow-running band

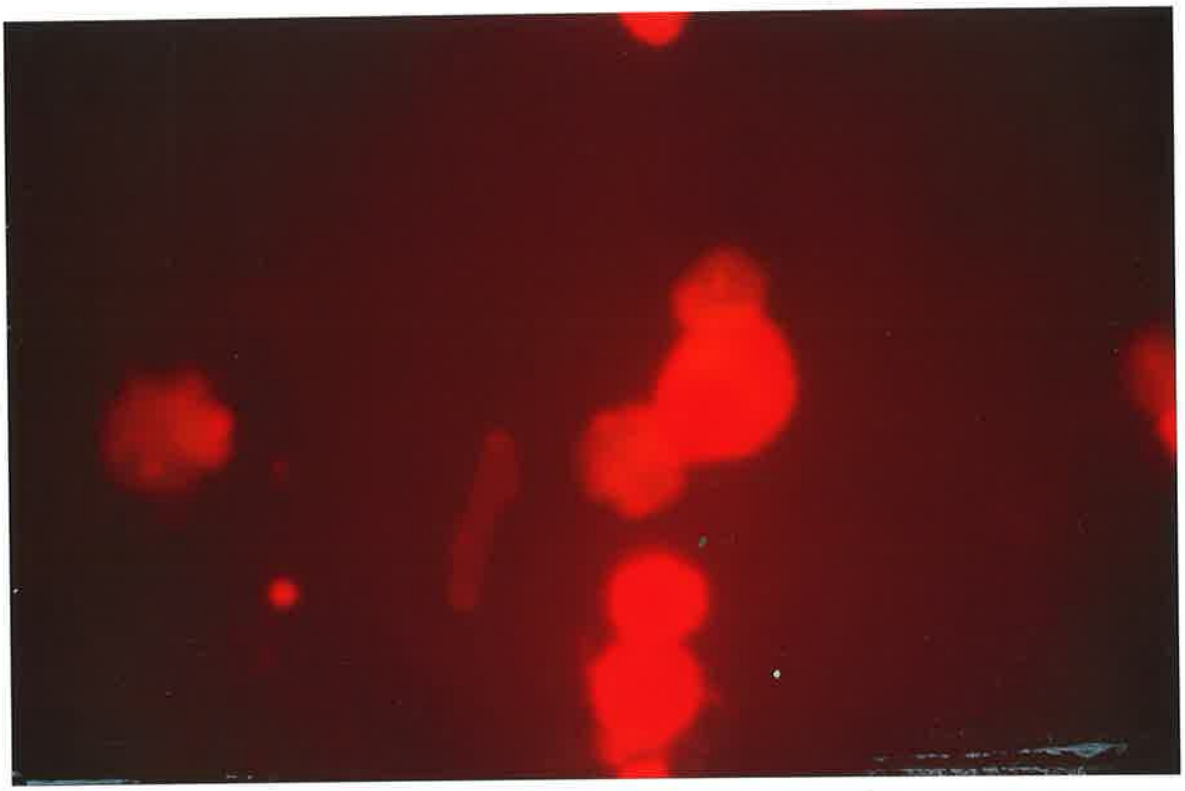


Fig. 4.12

Fluorescence uptake of HPD in Raji cells

Raji cells were incubated with HPD ($O.D._{397}=4.0$) for 1h and examined under a fluorescence microscope (400X magnification).

Table 4.2

Fluorescent uptake of porphyrin fractions by Raji cells

Porphyrin	Fluorescence
HPD	Pos
HPD retained fraction (YM10 ultrafilter)	Pos
HPD passed fraction (YM10 ultrafilter)	Weak Pos
HPD aggregate (p10 column)	Pos
HPD intermediate fraction (p10 column)	Pos
HPD slow fraction (p10 column)	Weak Pos
Protoporphyrin	Pos
Haematoporphyrin	Neg
Hydroxyethyl vinyl deuteroporphyrin	Pos

Cells were incubated for 1h in porphyrin ($O.D._{397}=4.0$) and examined under a fluorescence microscope.

incubated in HP were negative. This correlated with the order of photoactivity demonstrated by ^{51}Cr release and indicates that a crucial requirement for photoactivity may be uptake of the porphyrin into the cells.

(iv). Photocytotoxicity of porphyrins "external" to the cells.

Cells were suspended in porphyrin ($O.D._{397}=0.4$) and irradiated without any preincubation. HPD, HPD retained fraction (YM10 ultrafilter) and HVD all caused rapid ^{51}Cr release (Fig. 4.13). PP was slightly less active than HPD, HPD passed fraction (YM10 ultrafilter) was less active and HP was nearly inactive (Fig. 4.14). This order of activity was the same as that observed when porphyrins and cells were incubated for 1h before irradiation.

The location of the porphyrin could be either in the external medium, or during the time needed to irradiate the cells, it could attach to the cell surface or be taken up into the cells. Cells were suspended in HPD ($O.D._{397}=0.4$) and examined immediately under a fluorescence microscope. There was faint red fluorescence around the rim of the cells but none in the cytoplasm, indicating very rapid binding of porphyrin on the cell surface.

(v). Photocytotoxicity of porphyrins after extended incubation followed by efflux

^{51}Cr release after 21h incubation in porphyrin followed by efflux is shown in Fig. 4.15. HPD retained fraction (YM10 ultrafilter) was slightly more photoactive than HPD. HPD passed fraction (YM10 ultrafilter) was much less photoactive. Relative photoactivities were not altered by extended incubation of the cells in porphyrins. The extended incubation did not cause an inactive porphyrin to become phototoxic.

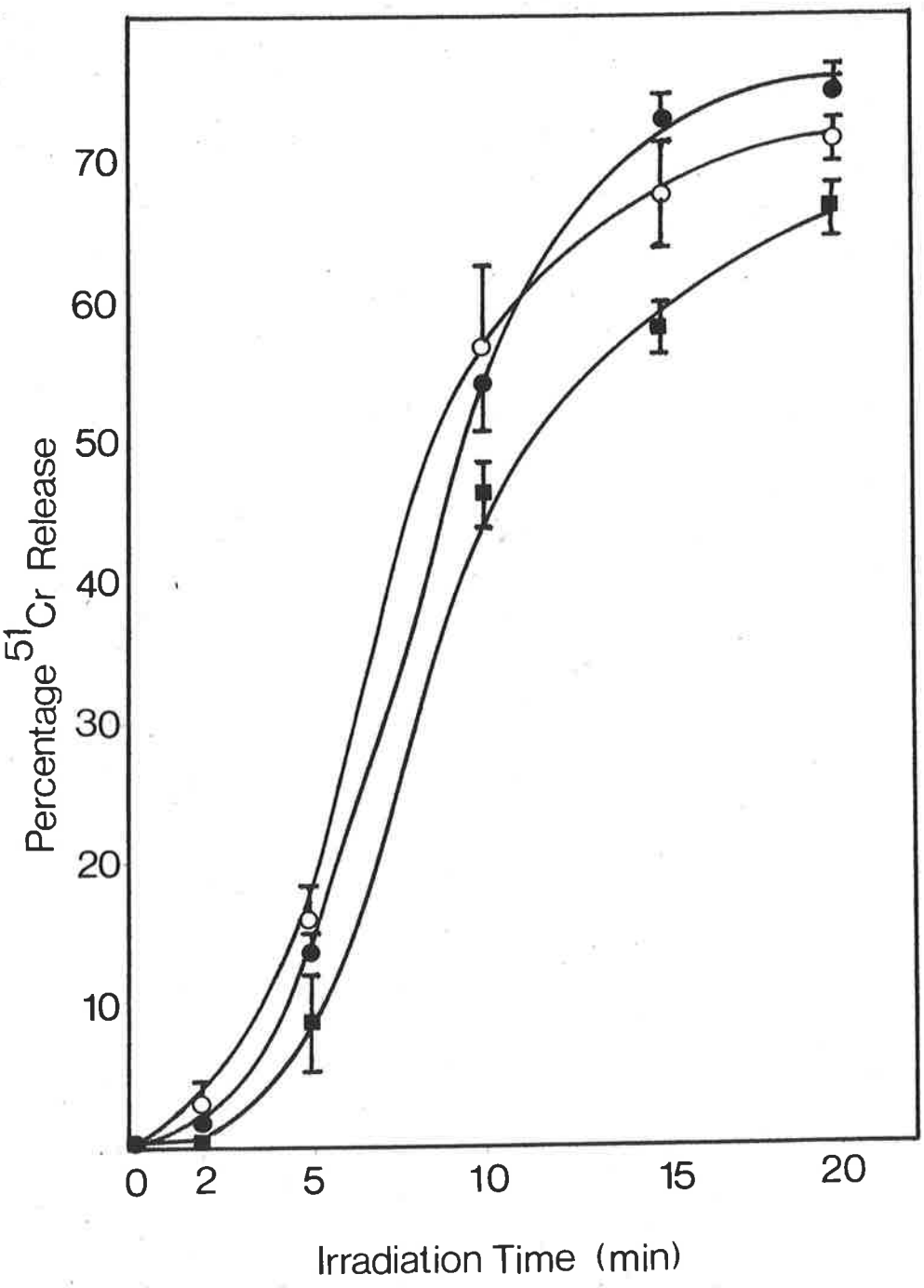


Fig 4.13

Photoactivity of porphyrins "external" to the cells

^{51}Cr-labelled Raji cells were suspended in porphyrin solutions (O.D.$_{397}$=0.4) and irradiated at once.

● HPD

○ HVD

■ PP

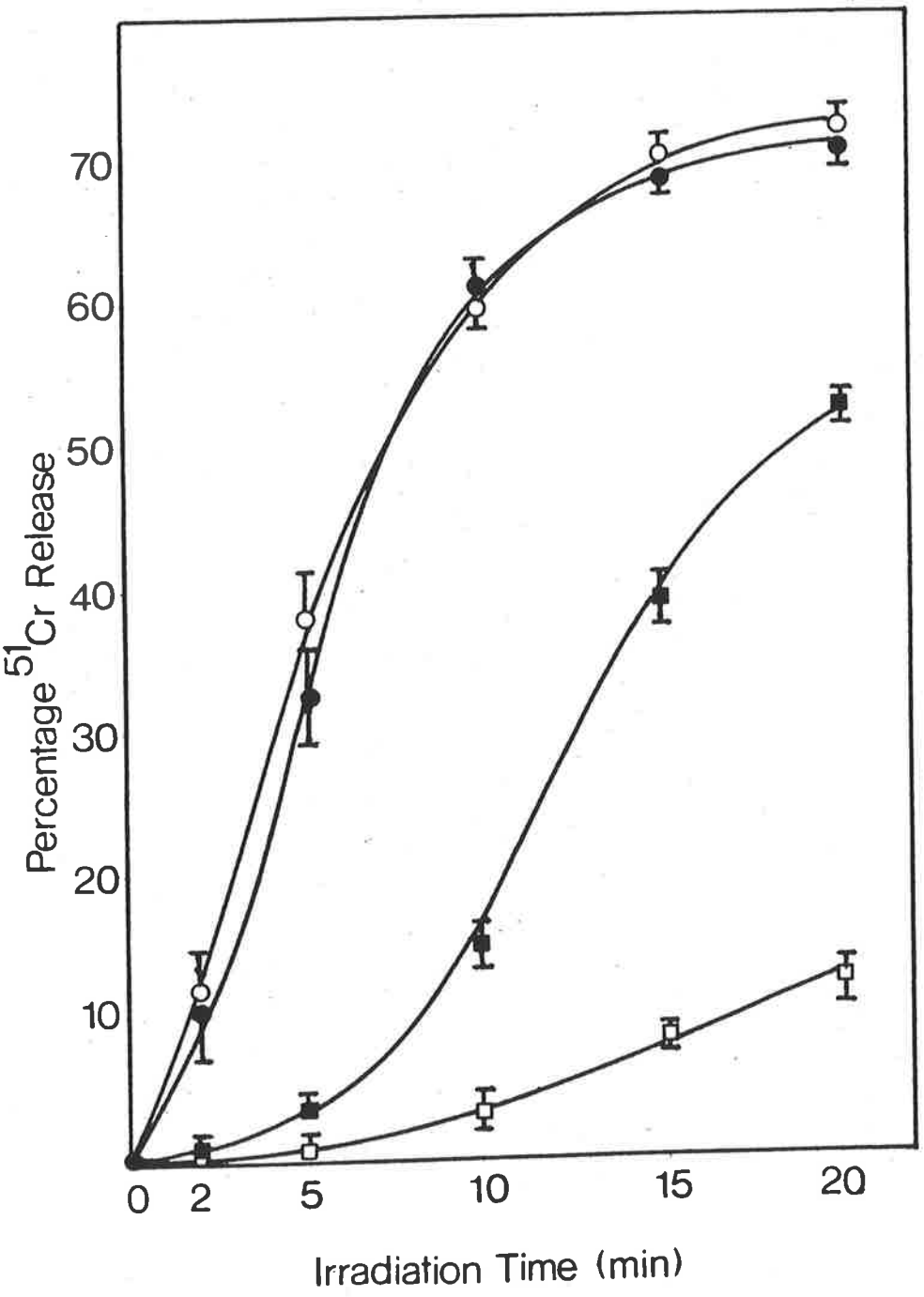


Fig. 4.14

Photoactivity of porphyrins "external" to the cells

^{51}Cr-labelled Raji cells were suspended in porphyrin solutions (O.D.$_{397}$=0.4) and irradiated at once.

- ● HPD
- ○ HPD retained fraction (YM10 ultrafilter)
- ■ HPD passed fraction (YM10 ultrafilter)
- □ HP

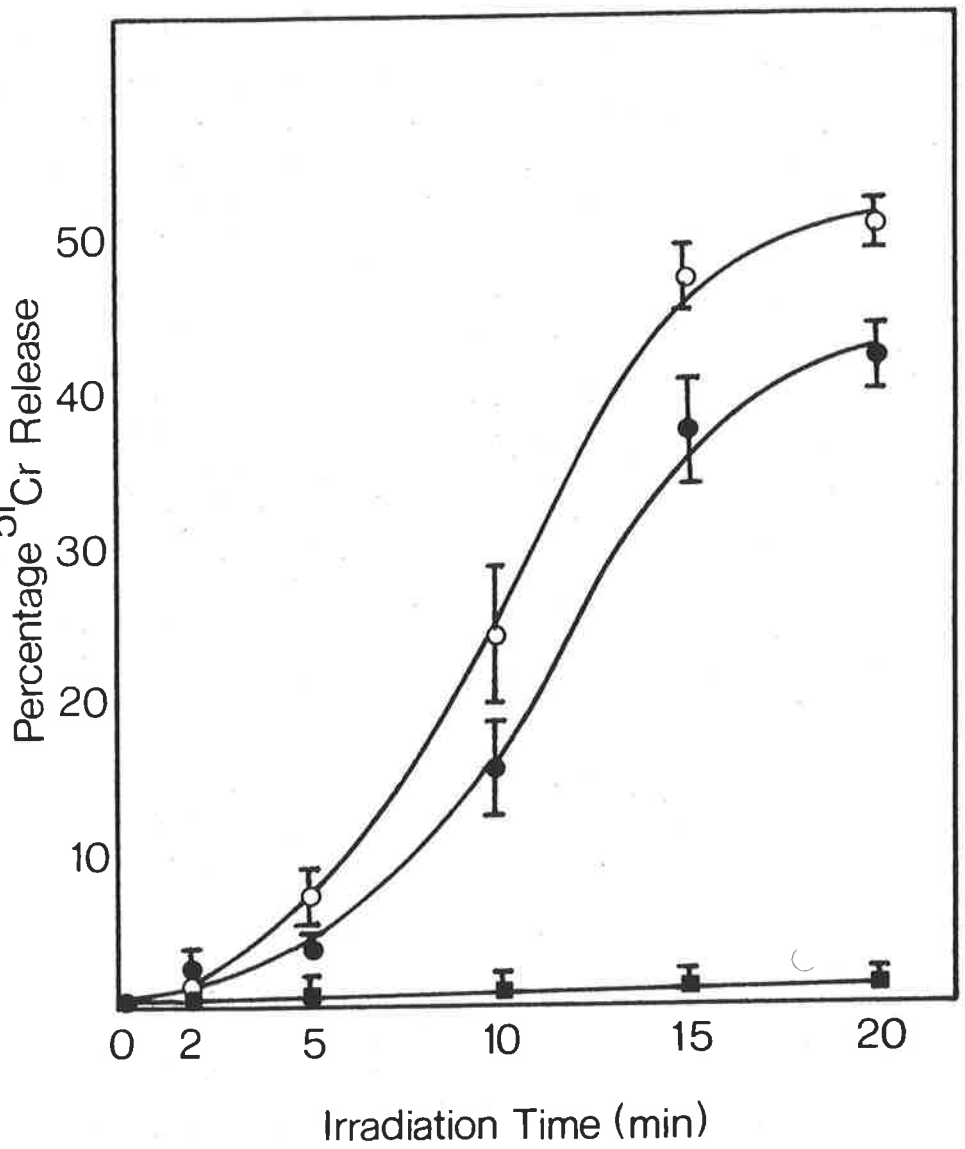


Fig. 4.15

Photoactivity of porphyrin fractions after extended incubation followed by efflux

Raji cells were incubated for 21 h with porphyrins ($O.D._{397}=4.0$), washed and incubated for 4h in fresh serum rich medium with ^{51}Cr. The cells were washed and irradiated.

- ● HPD
- ○ HPD retained fraction (YM10 ultrafilter)
- ■ HPD passed fraction (YM10 ultrafilter)

(vi). Photocytotoxicity of porphyrin C

In contrast to HPD, porphyrin C was not photoactive *in vitro*, even at very high porphyrin concentrations ($O.D._{397}$=60.0, equivalent to 350ug/ml) (Fig. 14.6). Fluorescence was not detected, suggesting porphyrin C was not taken up by the cells. Biogel p10 chromatography of porphyrin C showed a single peak which comigrated with HPD slow fraction, indicating porphyrin C was not highly aggregated.

(d). Discussion

The photocytotoxicity *in vitro* of some of the components of HPD has been assessed by ^{51}Cr release from Raji cells after irradiation. As discussed in Chapter 2, ^{51}Cr release was a measure of gross membrane damage leading to cell lysis, and correlated with trypan blue uptake. ^{51}Cr release, measured immediately after irradiation, was not as sensitive in detecting photodynamic damage to cells as inhibition of ^{3}H thymidine uptake or colony formation. However, it was satisfactory as a screening test for photoactivity *in vitro* of porphyrins with good reproducibility within an assay.

The concentrations of porphyrins used in the assays were determined from the measurement of $O.D._{397}$. Since the extinction coefficients vary between porphyrins [Barrett, 1969], there will be some inaccuracies in concentration. However the smallness of the quantities of porphyrin isolated from p10 columns or from electrophoresis made the determination of dry weight impractical.

Photoactivity of porphyrins was assessed by examining the rate of ^{51}Cr release. A number of porphyrin preparations had profiles of ^{51}Cr release similar to HPD. These were HPD

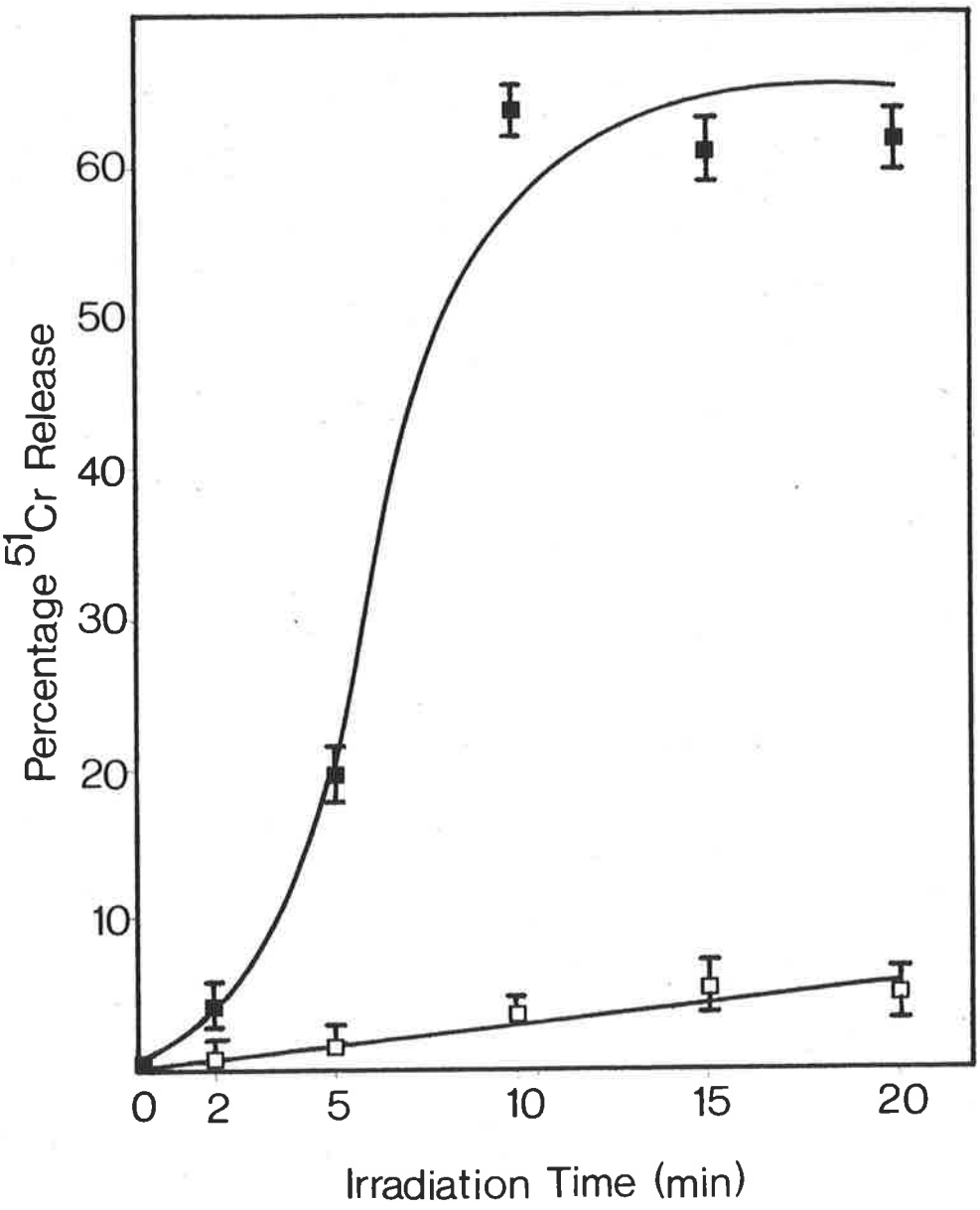


Fig. 4.16

Photoactivity of porphyrin C

^{51}Cr-labelled Raji cells were incubated for 1h in HPD or porphyrin C and irradiated.

■ HPD ($O.D._{397}$=4.0)

□ Porphyrin C ($O.D._{397}$=60.0)

aggregate, HPD retained fraction, PP and all the aggregation states of HVD. The HPD intermediate and HPD slow fractions were less active. HPD passed fraction, pure HP and porphyrin C (all less aggregated) were inactive. Thus the photoactivity of the porphyrins increased with increasing aggregation state.

These results are in agreement with other published reports. Pure HP was inactive in vitro [Kessel, 1982a, 1982b] and in vivo [Dougherty, 1983] and any photoactivity in commercial HP could be attributed to impurities. Kessel [1982a] showed L1210 cells preferentially accumulated the PP and HVD contaminants in commercial HP.

Studies by Moan and coworkers also confirmed the above results. The least polar components in HPD photosensitized NHIK 3025 cells in vitro but HP was not photoactive [Moan et al., 1982b; 1982c; Moan and Sommer, 1984]. The photosensitizing efficiency of the components of HPD increased with decreasing polarity, with a corresponding increase in uptake of porphyrin, measured both by fluorescence and ^{3}H-porphyrin uptake. Moan and Sommer [1983] reported that intermediate-sized hydrophobic porphyrins were accumulated in vitro rather than the large aggregates. They did not directly determine the size of porphyrins aggregates, but deduced it from the relative sharpness of the HPLC peaks.

Kessel has also confirmed that hydrophobicity is a major factor in determining photoactivity in vitro [Kessel, 1981; 1982a; 1982b]. Hydrophobic porphyrins were readily taken up by L1210 cells but were washed out of the cells. Hydrophilic porphyrins were slowly accumulated and were not readily washed out of cells. This contradicts the above results where HPD

passed fraction, a mixture of relatively hydrophilic porphyrins by HPLC, was not taken up or retained by Raji cells after long incubation. This discrepancy may reflect the cells and porphyrins used and also differences in sensitivity of the assays used to detect photodynamic damage to cells.

Moan and Sommer [1981] showed irradiation of HP resulted in the highest quantum yield of $^1O_2$ and the hydrophobic component the lowest. This is in the reverse order to the photoactivity observed in vitro, implying the quantum yield of $^1O_2$ is not a major factor in determining photocytotoxicity of porphyrins.

Photoactivity corresponded to fluorescence of porphyrins within the cells. Kessel [1977b] reported the ability of a porphyrin to bind to the cell surface was the most important factor in determining its photosensitizing ability. The intensity of fluorescence is affected by the nature of the porphyrin and the site of localization within the cell as well as the amount of porphyrins present. Porphyrin aggregates are not fluorescent [Moan and Sommer, 1981; Moan et al., 1983b]. Bright cytoplasmic fluorescence was observed after incubating cells in HPD aggregate, suggesting porphyrins disaggregate within cells. Therefore aggregation is required for uptake but the photoactive porphyrin within the cell may not be an aggregate. Moan and Sommer [1984] suggested non-aggregated porphyrins were more efficient photosensitizers within the cell, since the action spectrum of photodynamic inactivation closely corresponded to the fluorescence excitation spectrum of non-aggregated porphyrins. This is in agreement with Kessel [1982a] who found that the intracellular porphyrin was HP. Since HP is not taken up by cells, the disaggregation of an

aggregate containing HP may occur within the cell. The high quantum yield of $^1O_2$ after irradiation of HP suggests HP would be a effective photosensitizer once it is within the cell.

In conclusion, the requirements of a porphyrin for photocytotoxicity in vitro are hydrophobicity, possibly to allow binding to lipid-rich regions in cell membranes, and a highly aggregated state. It has been suggested HPD may be taken up by phagocytosis [Moan and Christensen, 1980]. This may occur more readily with large aggregates. The tumour localizing capacity and chemical structure of these large aggregates will be considered further in Chapter 5.

CHAPTER 5

PHOTOCYTOTOXICITY IN VIVO OF HPD COMPONENTS

(a). Introduction

The previous chapter discussed the *in vitro* photocytotoxic activity of some of the components of HPD. Photoactivity of porphyrins correlated with a high degree of aggregation and with the presence of more hydrophobic porphyrins. HP, which was hydrophilic and not highly aggregated, was inactive *in vitro*.

The photoactivity of porphyrins *in vivo* must also be considered. Tumour-localizing capacity will play a major role in determining the phototoxic activity of porphyrins. Since the tumour vasculature is an important site of photodynamic damage, interactions between porphyrins and the vasculature will also be important in determining phototoxicity.

The transplantable tumour model in mice described in Chapter 3 has been used to examine the phototoxic and skin-sensitizing capacities of some of the components of HPD.

(b). Methods

(i). Preparation of Porphyrins

Porphyrins were prepared and analysed as described in Chapter 4. Since HVD could not be prepared in large quantities by acid-ether extraction, testing *in vivo* was not possible. Photofrin II was a gift from Dr. T.J. Dougherty. All porphyrins were tested for purity by HPLC and for aggregation state by Biogel p10 chromatography before being administered to the mice. Concentration of porphyrins was determined by measuring $O.D._{397}$ in 1:1 Ethanol:0.1N NaOH and compared to HPD as a standard. HPD (5mg/ml determined by dry weight) when diluted

1:1000, had an $O.D._{397}$ of 0.8.

(ii). Mouse Tumour Assay

Mice with Lewis lung carcinoma were given porphyrins (50mg/kg i.p.) and the tumours irradiated 24h later with 225J/sq cm light from the incandescent lamp. TC_{50}, skin photosensitivity and uptake of porphyrins were assessed as described in Chapter 3.

(c). Tumoricidal Activity of Porphyrins

The relative tumoricidal activity of the porphyrins is shown in Table 5.1. HPD, HPD retained fraction (YM10 ultrafilter) and Photofrin II all showed similar photoactivity, with a TC_{50} of 5-6 days. HPD passed fraction (YM10 ultrafilter) and HP were inactive, having no visible effect on the tumours. PP had very little phototoxic activity but an occasional tumour disappeared after irradiation.

The photoactivity of porphyrin C was tested using a short interval between injection and irradiation, since fluorescence uptake studies (see below) showed porphyrin C was cleared very quickly from the mouse. The photoactivity of porphyrin C compared to HPD is shown in Table 5.2. When the tumours were irradiated 1h after injection of porphyrin C, TC_{50} was 4 days, comparable to the response if the tumours were irradiated 24h after injection of HPD. The efficacy of treatment diminished over the next hour with no response 2h after injection of porphyrin C. When tumours were treated 2h after HPD injection, TC_{50} was 8-9 days.

(d). Correlation between tumour fluorescence and phototoxicity

Twenty four hours after injection of porphyrin, there was

Table 5.1

In vivo photoactivity of porphyrins

Porphyrin	TC_{50} (days)	% Increase in Footpad Thickness	Fluorescence
HPD	5	79 ± 21	Pos
HPD retained fraction	5	69 ± 9.9	Pos
HPD passed fraction	0	0.8 ± 6.7	Weak Pos
HP	0	0 ± 2.0	Neg
PP	0	-0.2 ± 3.0	Neg
Photofrin II	6	55.4 ± 11.5	Pos

Mice were injected with porphyrin (50mg/kg i.p) and tumours irradiated 24h later with 225J/sq cm light. For skin photosensitivity, the left footpad was irradiated with 110J/sq cm. Results are expressed as the mean ± S.D. of 2 experiments. Fluorescence of frozen sections was assessed 24h after injection of porphyrin.

Table 5.2

Comparison of the photoactivity of HPD and porphyrin C

Porphyrin (50mg/kg)	Interval porphyrin to Irradiation (hours)	TC_{50} (days)	Fluorescence
Porphyrin C	1	4.0	Pos
Porphyrin C	1.5	*<1	Weak Pos
Porphyrin C	2	0	Weak Pos
HPD	2	8.5 ± 0.5	Pos
HPD	24	5.0	Pos

*: 4/10 tumours were non-palpable 24 h post irradiation.

Mice were injected with 50mg/kg porphyrin i.p. and tumours irradiated with 225J/sq cm light. Fluorescence of frozen sections of tumours was assessed after the same interval between porphyrin injection and irradiation as for photoactivity.

intense red fluorescence in tumours of mice injected with HPD, HPD retained fraction and Photofrin II (Table 5.1.). Tumours from mice injected with HPD passed fraction were weakly fluorescent. Injection of PP and HP did not result in any fluorescence in the tumours. Photoactivity corresponded to fluorescence in the tumours.

Uptake of porphyrin C was examined by removing tumours 1-24h after injection. Bright red fluorescence was seen 1h after injection but was greatly reduced in the later samples. No fluorescence was detected 24h after injection (Table 5.2). Photoactivity correlated with the tumour response to PDT.

(e). Skin Photosensitivity

The percentage increase in footpad thickness 24h after irradiation is shown in Table 5.1. HPD, HPD retained fraction and Photofrin II all caused approximately the same cutaneous photosensitivity while HPD passed fraction, HP and PP did not photosensitize the skin. Skin photosensitivity correlated with tumour response to PDT. Skin photosensitivity induced by porphyrin C 1h after injection was not reproducible, with some mice showing increases in footpad thickness of up to 50% and others showing no response.

(f). Discussion

The validity of TC_{50} as a measure of the efficacy of PDT has been discussed in Chapter 3. The relative photoactivities of some of the components of HPD have been investigated using this assay.

The photocytotoxic activity *in vivo* of HPD was limited to the fraction retained by YM10 ultrafilter. This fraction is similar to the HPD aggregate fraction isolated by Biogel p10

chromatography. This is in agreement with a number of reports that the aggregated hydrophobic fraction of HPD is entirely responsible for the photocytotoxic activity *in vivo* [Dougherty et al., 1983; Moan and Sommer, 1983; Evensen et al., 1984b; Kessel and Chou, 1983; Kessel and Cheng, 1985]. Pure HP and PP were inactive, as was HPD passed fraction which consisted of less aggregated material. Pure HP or PP did not localize in sarcoma 180 tumours in mice [Kessel, 1982b]. Berenbaum et al. [1982] also found that HP, PP and HVD did not localize in tumours and were not photoactive *in vivo*. Commercial HP was photoactive in a rat tumour [Tomio et al., 1983]. However this photoactivity may be accounted for by hydrophobic contaminants in commercial HP, similar in composition to HPD aggregate [Dougherty, 1983].

The photoactivity of the porphyrins correlated with uptake of porphyrin in the tumour as detected by fluorescence. This suggests that the critical requirement for photoactivity *in vivo* is uptake and retention of porphyrin in the tumours. HPD passed fraction, HP and PP were not taken up or retained in the tumours. PP was photoactive *in vitro* (Chapter 4), thus it can be taken up by cells in tissue culture but the mechanism for its uptake and retention in the tumour must be lacking in mice. Skin photosensitization only occurred with porphyrins that also resulted in tumour fluorescence and destruction. Therefore it is unlikely that a porphyrin will be found that is highly photoactive in tumours but does not photosensitize the skin. A photoactive porphyrin with a short retention time in the body may be the most useful method of minimising photosensitivity.

The commercial product, Photofrin II has been postulated to be the active component of HPD [Dougherty et al., 1983]. It exists in aqueous solution in a highly aggregated state, demonstrated by gel chromatography [Dougherty et al., 1983] and spectroscopic data [Poletti et al., 1984]. Dougherty reported Photofrin II had greater tumoricidal activity with less skin sensitization than HPD. Half the amount of Photofrin II was required to achieve similar tumour levels when compared to HPD [Dougherty et al., 1984a]. Kessel and Chou [1983] also showed an improved ratio of uptake in tumour compared to skin using an aggregated fraction of HPD. The above results show Photofrin II had similar photoactivity to HPD and HPD retained fraction (YM10 ultrafilter). This discrepancy may reflect the different tumour systems used. HPD, HPD retained fraction and Photofrin II all caused a similar degree of cutaneous photosensitivity. Gomer and Razum [1984] also reported HPD and Photofrin II caused comparable skin damage. Therefore using the photoactive fraction of HPD did not have any therapeutic advantages with the same efficacy of tumour destruction and skin photosensitivity as HPD.

The chemical structure of the photoactive fraction of HPD is still controversial. Using nuclear magnetic resonance (NMR) and mass spectra, Dougherty has proposed that the photoactive fraction of HPD (Photofrin II) consists of a number of isomers of an HP dimer joined by ether linkages [Dougherty et al., 1984a; 1984b]. Kessel has proposed alternative structures where HP molecules are joined by ester linkages [Kessel et al., 1985; 1986]. However the photoactive fraction of HPD is not a dimer in solution. Biogel p10 chromatography and ultrafiltration studies described in Chapter 4 suggest a larger aggregate.

Ultracentrifugation studies [Swincer et al., 1985] provided evidence for a molecular weight of greater than 20,000. Therefore if the active fraction is a porphyrin dimer, there must be a considerable degree of stable self-aggregation in aqueous solutions.

Analysis of HPD aggregate by NMR [Ward and Swincer, 1985] also suggested a polymeric structure, possibly of 30-40 porphyrin units. NMR studies gave no evidence as to the nature of the covalent bond between the porphyrins. HPD aggregates were stable in both aqueous and organic solvents and did not disaggregate upon dilution. They appear to have a structure quite different to the weak aggregates formed by HP or the large aggregates (molecular weight greater than 100,000) formed by protoporphyrin in aqueous solution [Swincer et al., 1985]. Ward and Swincer [1985] postulated that HPD aggregate is formed by stacking of the tetrapyrrol rings. This arrangement would be favourable for transport through the hydrophilic environment of the blood. The stacks may then unfold in the hydrophobic environment of the cell membrane and the linear arrangement of porphyrins will readily insert into the cell membrane where a nucleophilic group could cleave off individual porphyrins. These monomeric porphyrins will be fluorescent and may then migrate to more sensitive sites in the cell.

The requirements for a photoactive and tumour-localizing porphyrin therefore appear to be a relatively hydrophobic nature and a highly aggregated state. However porphyrin C does not match these requirements as it is not highly aggregated (Chapter 4). Porphyrin C is rapidly cleared from the body, so aggregation may only be necessary for long-term retention of

porphyrins in tumours.

Porphyrin C may be potentially therapeutically useful. Its short retention time in the body means that long term photosensitivity would not be a problem and adequate tumour responses may be achieved by treating the tumours shortly after porphyrin injection. Scourides et al., [1985] have reported good localization of porphyrin C in the tumours of mice 2h after injection with only very low levels of fluorescence in normal tissues. Therefore new porphyrins with different rates of uptake and retention may be useful in improving the efficacy of PDT.

CHAPTER 6

PHOTOCYTOTOXICITY IN VITRO AND ANTIBODY SPECIFICITY OF HAEMATOPORPHYRIN-ANTIBODY CONJUGATES

(a). Introduction

The use of drugs or radioisotopes bound to tumour-specific antibodies is being investigated for the detection of tumours or to improve selectivity of drug uptake. Labelling of tumour-specific monoclonal antibodies with ^{123}I or ^{131}I followed by external body scintigraphy, has been used sucessfully for imaging breast carcinoma and metastases from ovarian and gastrointestinal adenocarcinoma [Epenetos et al., 1982; Zalcberg et al., 1983]. Monoclonal antibodies targeted against tumours have also been used for therapy. ^{131}I-labelled monoclonal antibody was used to deliver high doses of radiation locally to metastatic ovarian carcinoma but with low doses to the rest of the body. A reduction in tumour mass was shown [Courtnay-Luck et al., 1984].

Complexes between chemotherapeutic drugs and anti-tumour antibodies may improve specificity and efficacy of treatment. Chlorambucil-antibody complexes were used to treat a transplantable mouse melanoma with a better therapeutic response than free chlorambucil [De Weger et al., 1982]. Monoclonal antibodies have also been used to deliver toxic molecules such as Ricin A, abrin and diphtheria toxin, to tumours without systemic toxicity [Neville and Youle, 1982; Raso, 1982; Moolten et al., 1982]. Reduction in size of animal tumours and antibody specific cytotoxicity *in vitro* was observed.

The coupling of porphyrin to antibody has the potential to

increase specificity of uptake of porphyrin into tumours, thereby reducing the photosensitization of normal tissues. Mew and coworkers coupled HP to a monoclonal antibody directed against the mouse DBA/2J myosarcoma M-1. They showed that the conjugate was phototoxic against erythrocytes and exhibited antibody-directed specificity of uptake into mouse tumours [Mew et al., 1983; Wat et al., 1984]. The growth of tumours was inhibited by treating with HP-antibody and light.

The potential of HP-antibody conjugates to deliver porphyrins to tumours was examined as a method of increasing specificity of uptake and hence increasing efficacy of treatment. As an in vitro model, HP was coupled to polyvalent rabbit antihuman immunoglobulin and the antibody specificity and photosensitizing ability of the conjugate was tested against normal human lymphocytes.

(b). Methods

(i). Preparation of HP-antibody conjugate

The coupling method described by Mew et al. [1983] was used. Haematoporphyrin dihydrochloride (HP, 20mg) (Roussel) was dissolved in 1.25ml water and 0.8ml N,N-dimethylformamide (Sigma) and 20mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCl, Sigma) in 0.6ml water was added. After 30min at room temperature, 15mg rabbit antihuman immunoglobulin (5mg/ml, Dakopatts) was added. The solution was stirred at room temperature for 5h and the pH maintained between 6 and 7 with 0.1N HCl or 0.1N NaOH as appropriate. Monoethanolamine (50ul, Sigma) was added and the reaction mixture incubated overnight at room temperature. The solution was then dialysed against 0.001N phosphate buffer, pH7.4 for 4 days with 3 changes of

buffer per day. Dialysis overnight was against PBS, pH7.4.

The dialysed solution was freeze dried, redissolved in 3ml PBS and passed through a Sephadex G25 column to remove unconjugated HP. The column was eluted with PBS (flow rate 0.5ml/min), 2min fractions collected and $O.D._{397}$ (porphyrin) and $O.D._{280}$ (protein) measured. The porphyrin and protein peaks coincided and the peak fractions were pooled. Visible/U.V. spectroscopy of the conjugate (Varian 635 spectrophotometer) showed a protein peak at 280nm with the characteristic porphyrin spectrum (Soret band at approximately 380nm and smaller peaks at 500, 530, 570 and 630nm, Fig. 6.1).

Protein concentration (determined spectrophotometrically) was 2.6mg/ml. Porphyrin concentration was 46.5ug/ml (determined spectrophotometrically as described in Chapter 4, by taking absorbance at 397nm of a standard HPD solution of 5ug/ml as 0.8). The ratio of HP:antibody protein was 17.9ug HP/mg protein. A second preparation of HP-antibody conjugate had a protein concentration of 4.8mg/ml and porphyrin concentration of 100ug/ml. The ratio of HP:antibody was 20.8ug HP/mg protein.

To assess the effect of the carbodiimide coupling procedure on antibody specificity, rabbit antihuman immunoglobulin was passed through the coupling procedure as described above in the absence of HP. This solution was designated "antibody control". The protein concentration of the antibody control was 0.66mg/ml.

(ii). Thin layer chromatography of HP-antibody conjugate

HP-antibody conjugate was run on Merck silica gel thin layer chromatography (TLC) sheets without fluorescence indicator with eluting solvent ethanol:acetic acid 95:5 v/v.

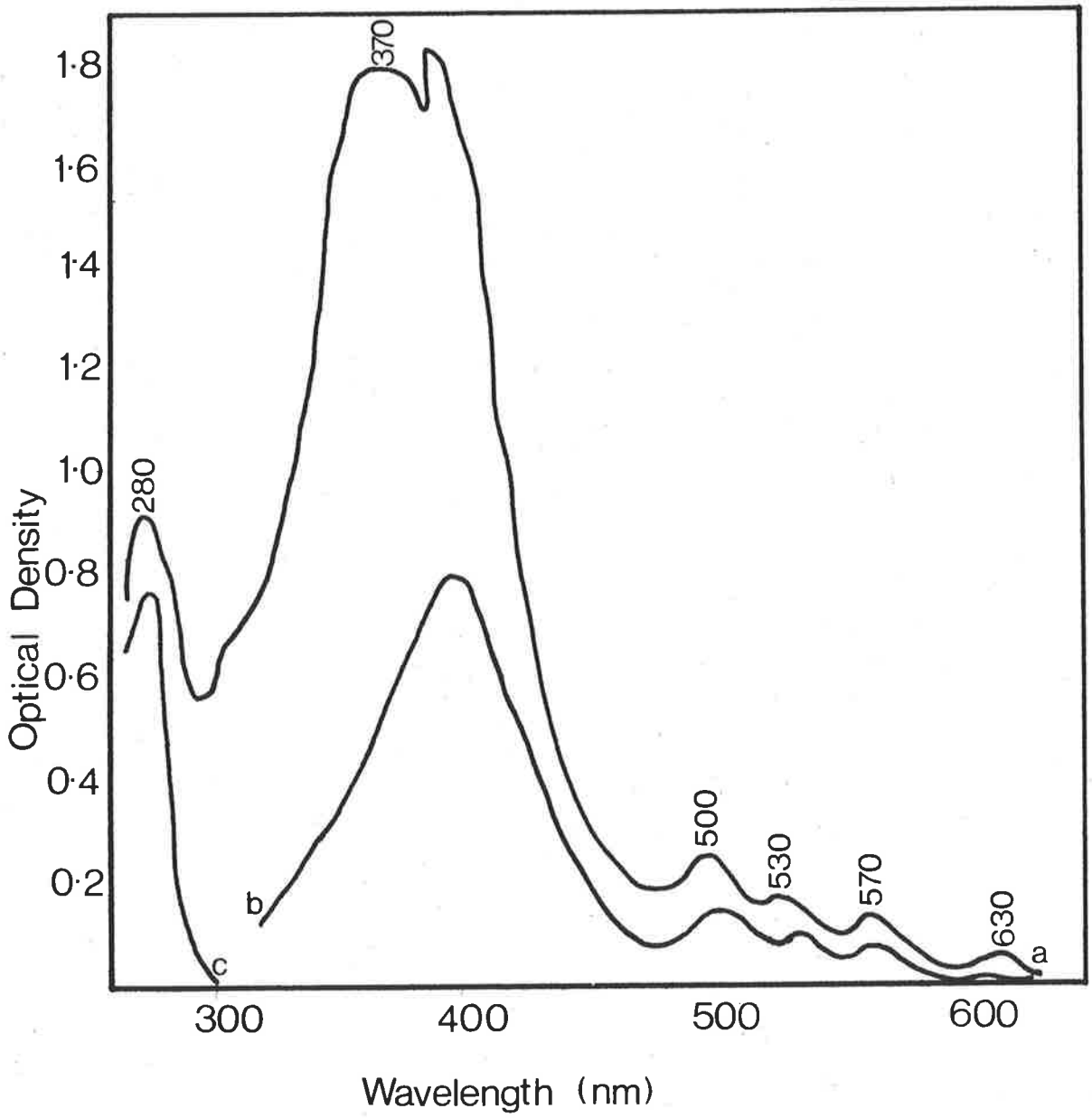


Fig. 6.1

Visible / U. V spectrum of HP-antibody conjugate

a: HP-antibody conjugate (11.2ug/ml HP, 0.7mg/ml protein)

b: HP (5ug/ml)

c: Antibody control (0.66mg/ml)

The plates were viewed under a ultraviolet lamp.

(iii). Antibody specificity of HP-antibody conjugate

Lymphocytes were isolated from heparinised blood from normal volunteers by Ficoll-Hypaque density gradients [Boyum, 1968]. The lymphocytes were washed in PBS, resuspended in PBS/10%FCS and incubated for 1h at 37^{o}. After washing, the cells were suspended in PBS at $5X10^{6}$/ml. The percentage of B cells was determined by incubating lymphocytes (100ul) with fluoresceinated $F(ab)_2$ polyvalent goat antihuman immunoglobulin (1.2mg/ml, Kallestadt) for 1h on ice [Forbes et al., 1978]. After washing in PBS, the cells were resuspended in FCS (20ul) and percentage of fluorescent cells counted under a Zeiss fluorescence microscope.

T cells were isolated by incubating lymphocytes ($10X10^{6}$/ml) for 1h at 37^{o} with 10% sheep red blood cells in PBS [Divakaran and Wangel, 1983]. The sheep red blood cells were pretreated for 15min at 37^{o} with a sulphydryl reagent, aminoethyl isothiuronium bromide (AET) at a ratio 1:4 packed sheep red blood cells:0.143M AET, pH9.0 [Kaplan and Clark, 1974]. The rosetted cells were resuspended and run on a Ficoll-Hypaque density gradient. The pellet contained rosetted T cells. Sheep red blood cells were lysed from rosettes by suspending the pellet in 1ml water for 20 sec before adding 20 ml PBS. T cells were resuspended in PBS ($5X10^{6}$/ml). Percentage T cells was determined by rosetting for 1h with 0.5% AET-treated sheep red blood cells [Forbes et al., 1978] and percentage B cell contaminant was determined as described above.

Lymphocytes (100ul) were incubated for 1h on ice with graded concentrations of HP-antibody in PBS, "antibody control"

or untreated antibody. Total final volume was 200ul. The cells were washed three times in PBS, resuspended in fluoresceinated swine antirabbit immunoglobulin (25ul, Dakopatts) and 50ul PBS, and incubated for 30min on ice. The cells were washed three times in PBS, resuspended in 20ul FCS and percentage of fluorescent cells counted.

(iv). Photoactivity of HP-antibody conjugate

Lymphocytes (100ul) were incubated for 1h on ice with HP-antibody or antibody control (4.5ug/ml HP, 390ug/ml antibody protein). This concentration of conjugate resulted in maximum binding to lymphocytes (see results of Hp-antibody binding studies in Fig. 6.3). The cells were washed three times and resuspended in PBS ($0.5X10^6$/ml). Cells were irradiated with white light from a Leitz projector with a 250W quartz halogen lamp. At approximately 5min intervals, 50ul aliquots were removed, added to 50ul of 0.3% trypan blue and percentage trypan blue-positive cells counted.

(v). Binding of HP-antibody conjugate to Lewis lung carcinoma cells

Lewis lung carcinoma cells were grown *in vitro* and harvested as described in Chapter 3. Binding and photocytotoxicity of the HP-antibody conjugates were tested as described above using the same concentrations of HP-antibody conjugate as for lymphocytes.

(vi). Blocking HP-antibody binding with free antibody

Lymphocytes (100ul) were incubated for 1h on ice with 2mg/ml $F(ab)_2$ goat anti human immunoglobulin (Kallestadt), washed three times with PBS, resuspended in PBS (100ul) and labelled with graded concentrations of HP-antibody conjugate as

described above. After incubation with fluoresceinated antirabbit immunoglobulin second antibody, the percentage of fluorescent cells was counted.

(vii). Blocking HP-antibody binding with free HP

Lymphocytes (100ul) were incubated for 1h at 37^{o} with graded doses of unconjugated HP (0-100ug/ml), washed, resuspended in PBS (100ul) and labelled with HP-antibody or antibody control as described above. Percentage of fluorescent cells was counted.

(c). Results

(i). TLC of HP-antibody conjugate

Examination of unconjugated HP on TLC showed an intense red fluorescent spot (Rf=0.85) with a fainter spot that remained on the origin. HP-antibody conjugate showed an intense red fluorescent spot at the origin only, indicating that unbound HP was a very minor contaminant in the conjugate.

(ii). Binding of HP-antibody conjugate to normal lymphocytes

When normal lymphocytes were incubated with HP-antibody, there was a linear increase in the percentage of fluorescent cells (Fig. 6.2), indicating that HP-antibody bound to all the lymphocytes rather than only to the B cells. This suggests a lack of antibody specificity. In a second experiment (Fig. 6.2), there was a plateau at 45% fluorescent cells. The antibody control (passed through the coupling procedure) gave 20% fluorescent cells (Fig. 6.2). B cell percentage was 18%, indicating the coupling procedure itself did not alter the antibody specificity.

(iii). Binding of HP-antibody to T cells

Surface marker analysis of the isolated T cell population

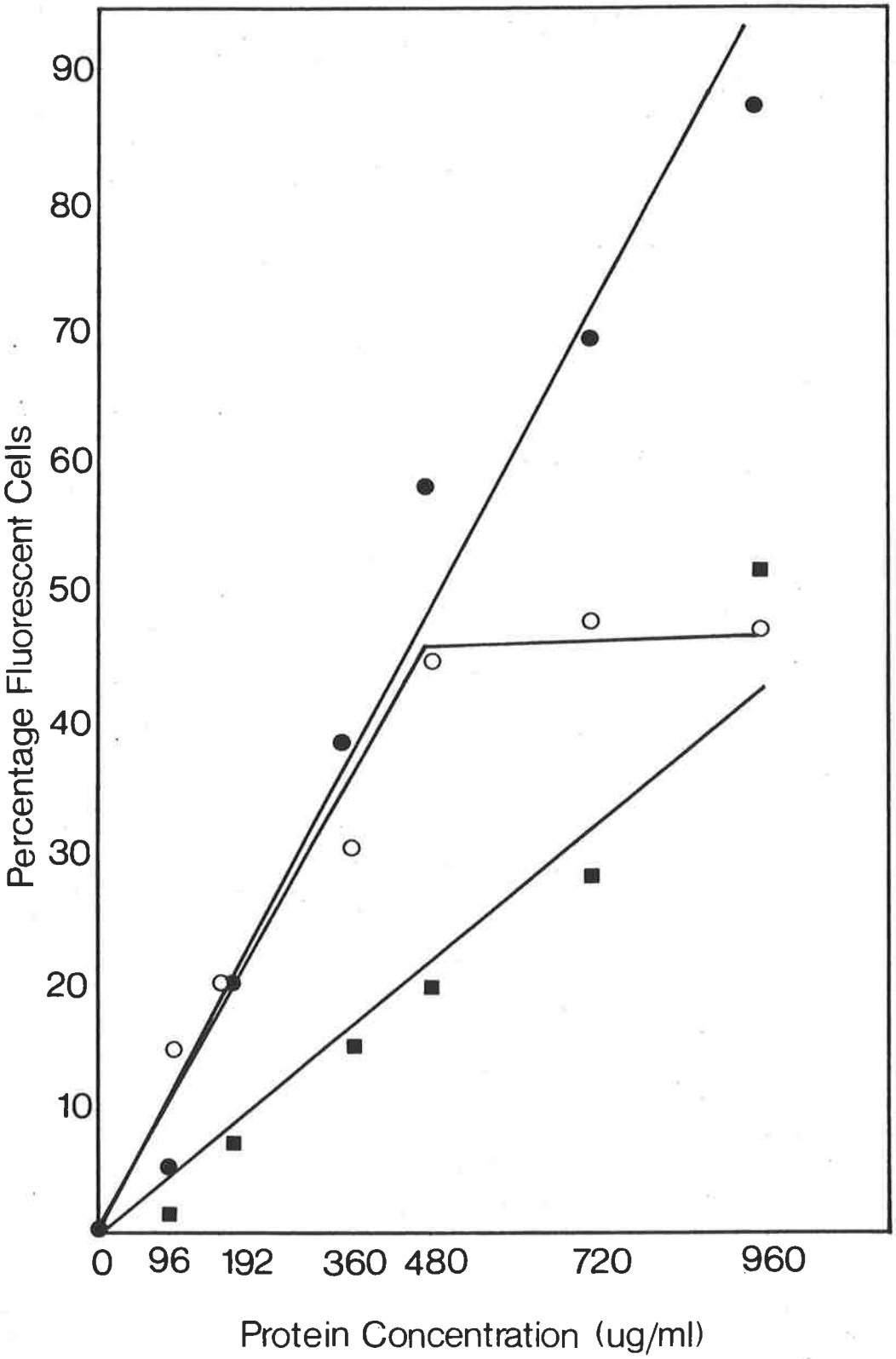


Fig. 6.2

Binding of HP-antibody to lymphocytes or T cells

Cells were incubated for 1h in graded doses of HP-antibody, labelled with fluoresceinated swine antirabbit immunoglobulin second antibody and percentage fluorescent cells counted.

- ● HP-antibody-labelled lymphocytes (expt. 1)
- ○ HP-antibody-labelled lymphocytes (expt. 2)
- ■ HP-antibody-labelled T cells

showed 92% AET sheep red blood cell rosettes (T cells) and 3% surface immunoglobulin positive cells (B cells). Labelling of the T cells with increasing concentrations of HP-antibody resulted in 40% fluorescent cells (Fig. 6.2),indicating T cells also bound HP-antibody conjugate. This confirmed the lack of antibody specificity of HP-antibody conjugate.

(iv). Photocytotoxicity of HP-antibody conjugate

The photodynamic activity of HP-antibody conjugate is illustrated in Fig. 6.3. Cell death was proportional to light dose, with 100% of the cells being killed after 35min irradiation. However, only 15 and 35% of the lymphocytes from experiments 1 and 2 were fluorescent after labelling with HP-antibody followed by fluorescent antiimmunoglobulin. This indicated some cells were killed which did not have detectable HP-antibody bound to the surface.

(v). Interactions between HP-antibody and Lewis lung carcinoma cells

All Lewis lung carcinoma cells bound HP-antibody, demonstrated by 100% fluorescent cells. However the cells were negative for surface immunoglobulin and did not bind antibody control, confirming the lack of antibody specificity of HP-antibody conjugate.

The photodynamic activity of HP-antibody bound to Lewis lung carcinoma cells is shown in Fig. 6.4. All the cells had been killed after irradiation for 50min.

(vi). Blocking HP-antibody binding with free antibody

Preincubation of lymphocytes with $F(ab)_2$ goat antihuman immunoglobulin blocked binding of fluoresceinated antihuman immunoglobulin to B cells, indicating that the surface immunoglobulin on the B cells was saturated by antibody (Table

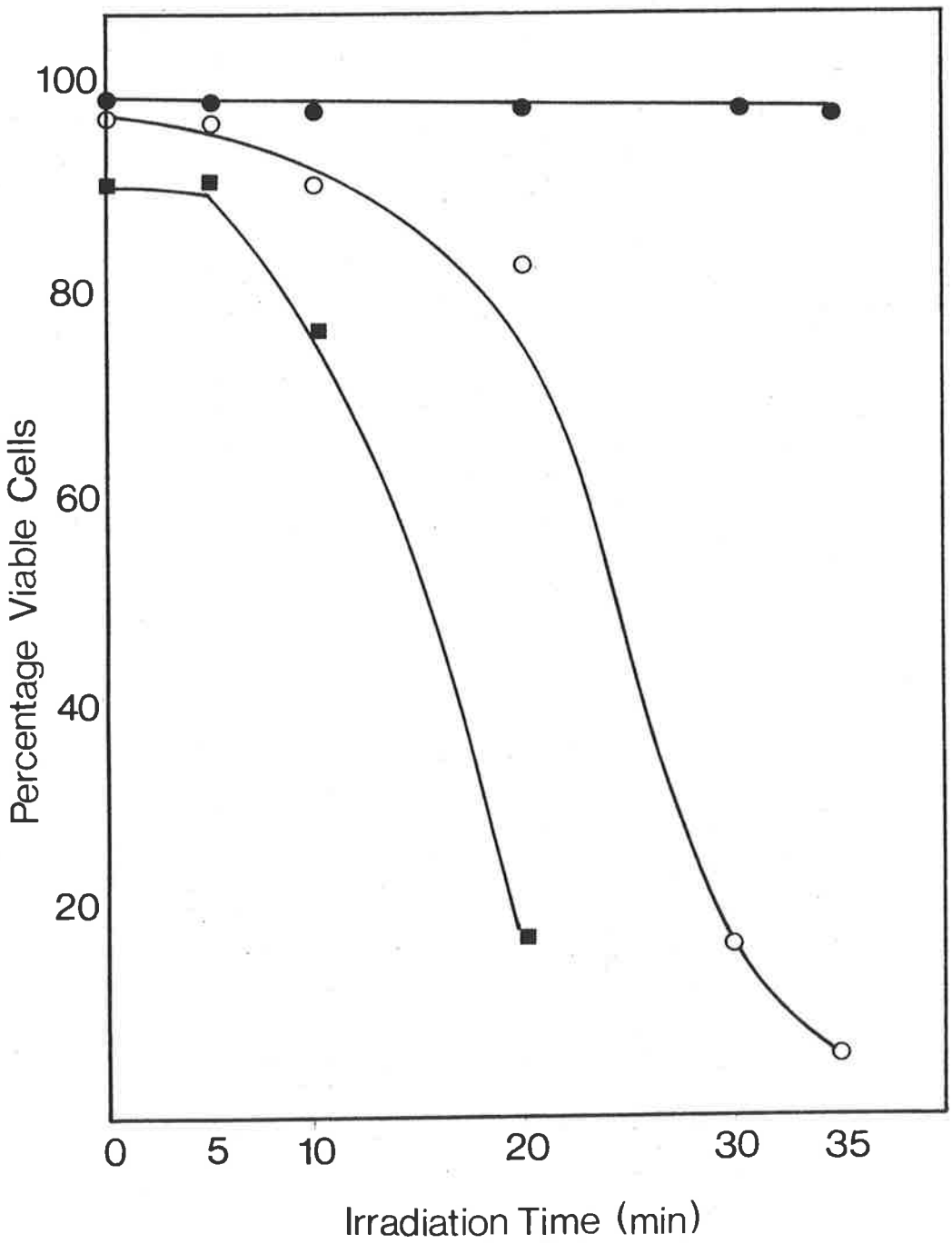


Fig. 6.3

Photocytotoxicity of HP-antibody conjugate

Lymphocytes from two subjects were incubated with HP-antibody (4.5ug/ml HP) or antibody control and irradiated. Percentage trypan blue-positive cells was counted.

○ HP-antibody-labelled lymphocytes (expt. 1)

■ HP-antibody-labelled lymphocytes (expt. 2)

● Antibody control-labelled lymphocytes

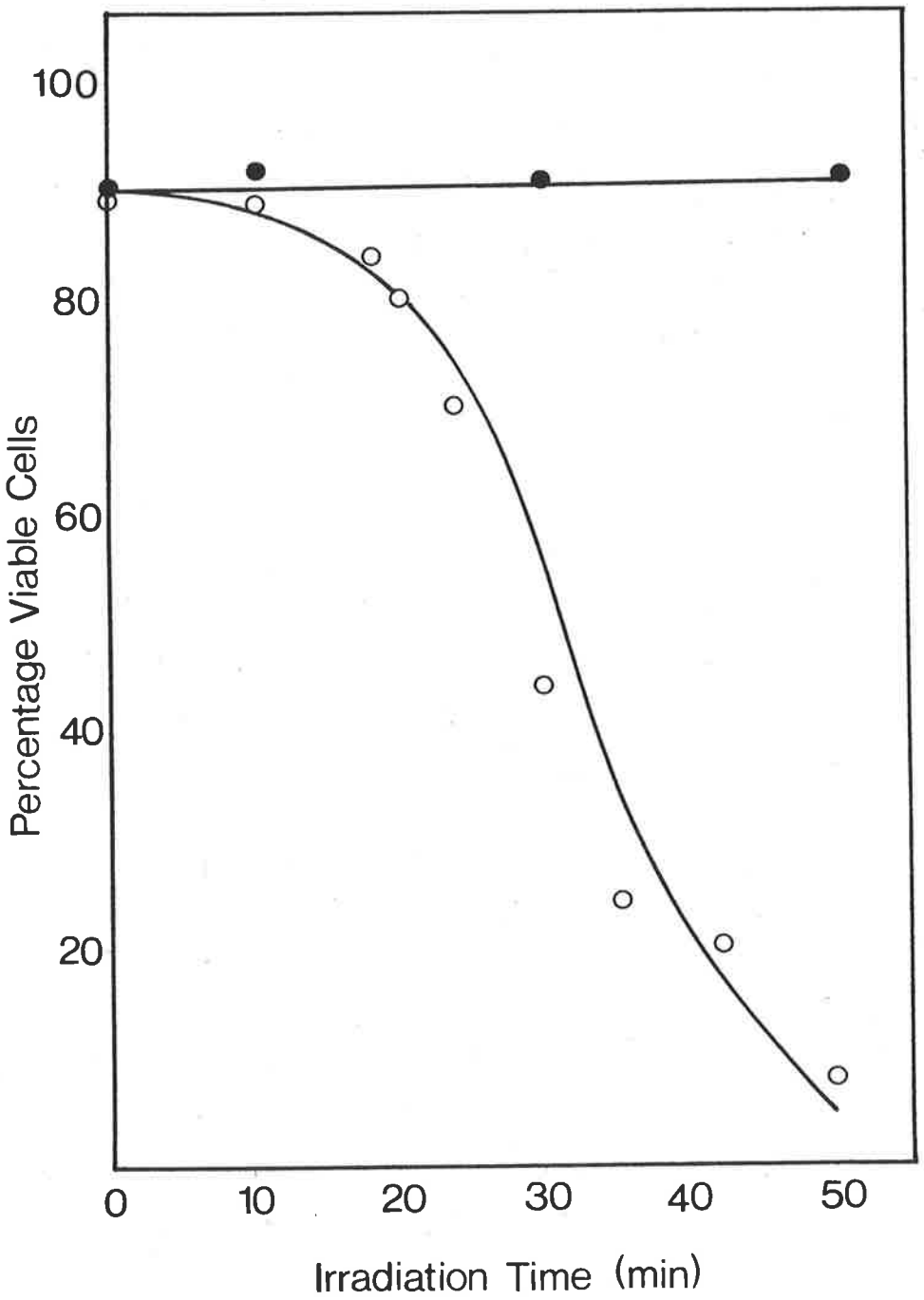


Fig 6.4

Photocytotoxicity of HP-antibody bound to Lewis lung carcinoma cells

Cells were incubated in HP-antibody (4.5ug/ml) and irradiated. Percentage trypan blue positive cells was counted.

- O HP-antibody-labelled Lewis lung carcinoma cells
- ● Antibody control-labelled Lewis lung carcinoma cells

6.1). However, preincubation with free antibody did not block binding of HP-antibody to lymphocytes. Therefore, HP-antibody does not bind to the cells through surface immunoglobulin (the specific antibody receptor).

(vii). Blocking HP-antibody binding with free HP

Preincubating lymphocytes in free HP had no effect on binding of HP-antibody (Fig. 6.5). Binding of control antibody to the lymphocytes was also unaffected by preincubating with HP. The proportion of fluorescent cells remained at 20%.

(d). Discussion

The use of tumour-specific antibodies to target the delivery of porphyrins into tumours has great potential to improve specificity of uptake of porphyrins by tumours while avoiding photosensitization of normal structures. It is therefore important to determine if this method can be used for improving the efficacy of PDT.

HP was covalently coupled to a polyvalent rabbit antihuman immunoglobulin antibody, using carbodiimide which forms an amide bond between free NH_2 groups on the antibody protein and carboxyl groups on HP. Unconjugated HP was removed by dialysis followed by Sephadex G25 column chromatography. No free HP was detected by TLC.

The carbodiimide coupling process does not modify the structure of the antibody such that specificity for surface immunoglobulin on B cells is lost. The same percentage of fluorescent cells was detected using untreated antibody and antibody passed through the coupling procedure without HP.

When lymphocytes were incubated with graded doses of HP-antibody and then labelled with a fluorescent second antibody,

Table. 6.1

Blocking of HP-antibody binding by preincubation with free antibody

HP-antibody Protein Concentration (mg/ml)	% Fluorescent Cells	
	Lymphocytes	T Cells
0	0	0
1.2	19	5
2.4	44	20
4.8	45	51
Antibody Control	14	6
FITC-$F(ab)_2$ Anti Ig	1	0

Lymphocytes or T cells were preincubated in unconjugated antihuman immunoglobulin before labelling with HP-antibody, antibody control (passed through the coupling procedure) or FITC-$F(ab)_2$ antihuman immunoglobulin.

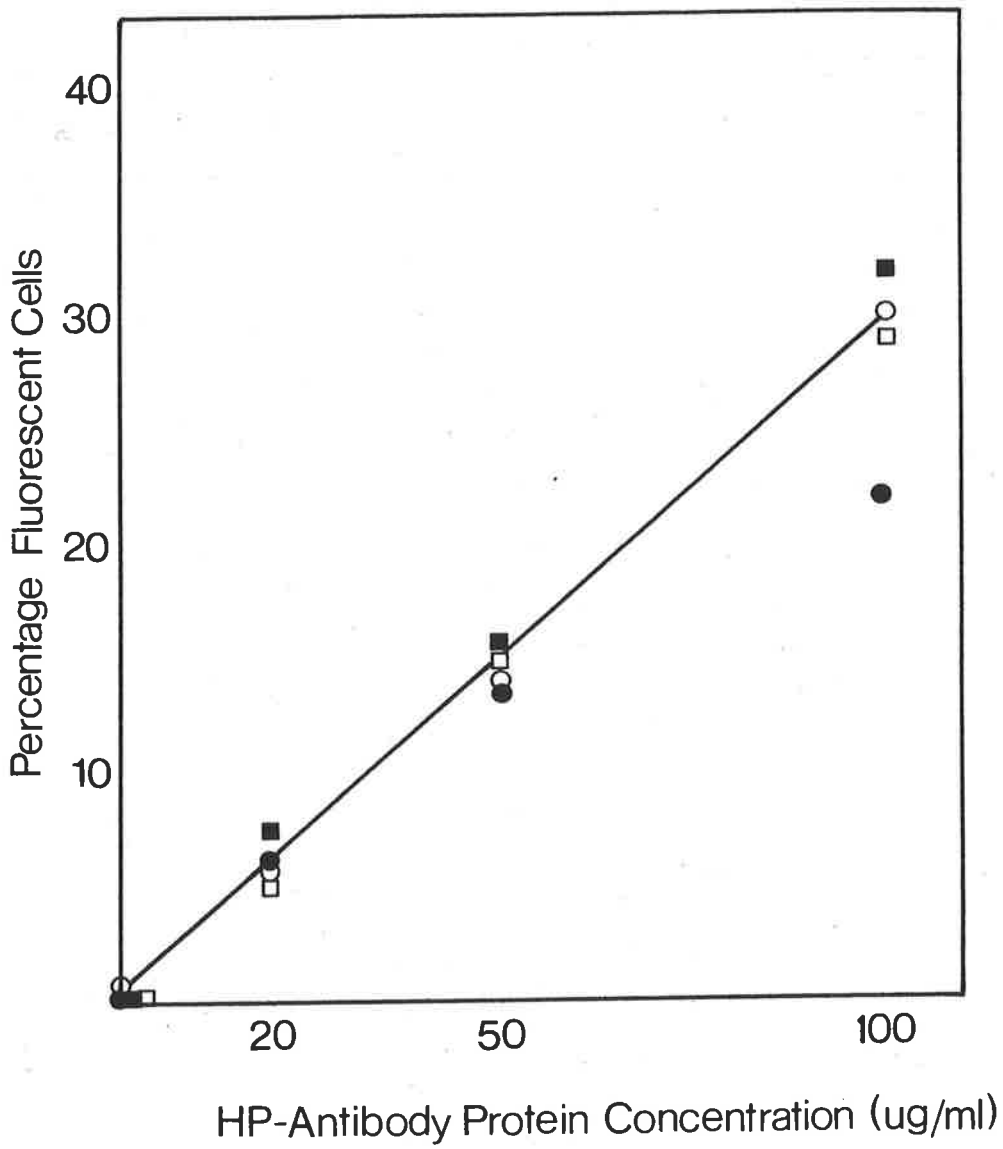


Fig. 6.5

Effect of preincubating lymphocytes with HP before labelling with HP-antibody conjugates

Lymphocytes were incubated with 0-100ug/ml HP, washed and labelled with HP-antibody. Percentage fluorescent cells was counted.

- ■ No HP
- □ 10ug/ml HP
- ○ 50ug/ml HP
- ● 100ug/ml HP

there was a linear increase in the percentage of fluorescent cells until most cells were labelled. Isolated T cells and Lewis lung carcinoma cells also bound HP-antibody, confirming that HP-antibody bound to cells lacking surface immunoglobulin. HP-antibody therefore displays a lack of antibody specificity.

The possibility that HP-antibody could be binding to lymphocytes by the F_c receptor for IgG rather than by surface immunoglobulin was considered. This was unlikely, since the percentage of HP-antibody positive cells in both T cells and lymphocytes was higher than the percentage of F_c-positive lymphocytes in normal blood (approximately 15%) [Forbes et al., 1978].

Uptake of HP-antibody could also be mediated by a receptor for porphyrins on the cell membrane. Preincubating lymphocytes in free HP had no effect on the binding of HP-antibody. However, pure HP was not taken up to any extent by cells in vitro (Chapter 4). Commercial HP was used for the above experiments. The hydrophobic contaminants in commercial HP will bind to cells [Kessel, 1982a] so any hypothetical porphyrin receptors could be at least partially blocked. HP-antibody conjugates may also be taken up non-specifically into cells by phagocytosis. It has been suggested that HPD uptake is partially mediated by phagocytosis [Moan and Christensen, 1980].

HP retained its photocytotoxic properties when covalently coupled to an antibody. Lymphocytes from two subjects had detectable HP-antibody only on 15 and 35% of the cells respectively, but all the cells were killed after irradiation. This could be due to several mechanisms. The phototoxic agent

(probably $^1O_2$), may diffuse from one cell to another. HP-antibody, released when the cells die, may bind to and photosensitize other cells. A further alternative is that low concentrations of HP-antibody bound to all cells but was not detectable by the fluorescent second antibody and was sufficient for a phototoxic reaction.

A commercial polyclonal antibody preparation will contain considerable quantities of contaminating proteins such as albumin, all of which will be conjugated with HP and may bind to the cells. However, these HP-labelled contaminants will not be detected by the fluoresceinated antiimmunoglobulin second antibody and will not contribute to the unexpectedly high percentages of fluorescent cells. The binding of these HP-labelled protein contaminants to lymphocytes would explain the photodynamic destruction of all cells when only a proportion had detectable HP-antibody on the surface.

HP was coupled to a monoclonal anti T cell antibody through the carbohydrate moiety on the F_c region with retention of photodynamic activity and antibody specificity [Oseroff et al., 1985a; 1985b]. Oseroff comments that the random binding of HP to amino acids which occurs in carbodiimide coupling would not be expected to conserve antibody specificity because of major alterations in antibody conformation. The above experiments show that the coupling procedure itself does not alter antibody specificity, but when HP is coupled, antibody specificity is lost, possibly due to major alterations in antibody shape or structure.

The discrepancy between the antibody specificity demonstrated by Mew et al. [1983] and the above results is not easily explained. It may be due to the different antibodies

used. A monoclonal antibody may be of higher purity than the polyclonal antibody used above. However Mew used diluted ascites as the monoclonal antibody preparation. This would be expected to contain albumin and other contaminating proteins, all of which would be coupled to HP. Antibody purity appears not to be essential for specificity of binding of HP-antibody.

The inability to demonstrate antibody-specific binding of HP-antibody conjugates implies that there are major problems in this method of directing porphyrins into tumours. However, the successful antibody-guided delivery of porphyrin into tumours by other authors suggests this method should be further investigated.

CHAPTER 7

PHARMACOLOGICAL MODULATION OF PDT WITH CYTOTOXIC DRUGS

(a). Introduction

Many of the patients being considered for PDT may be concurrently treated with cytotoxic drugs or undergo subsequent therapy. Therefore, interactions between cytotoxic drugs and PDT should be investigated to allow planning of more effective treatments. These interactions may either enhance or inhibit the efficacy of PDT. Better tumour destruction could be achieved or alternatively, lesser doses of cytotoxic drugs could be used with a corresponding reduction in morbidity. An additional benefit would be the reduction of cutaneous photosensitivity if less HPD were required for an adequate treatment. Interactions between PDT and cytotoxic drugs may also give some clues as to the mechanism of tumour destruction by PDT.

Studies were therefore undertaken, both *in vitro* and *in vivo*, to examine the effect of PDT in combination with six drugs used frequently for cancer chemotherapy, each representing a major group of cytotoxic drugs.

(b). Concurrent administration of HPD and cytotoxic drugs before PDT

Methods

The transplantable tumour model in mice described in Chapter 3 was used to assess the effects of cytotoxic drugs on the efficacy of PDT. Mice with Lewis lung carcinoma were given HPD (30mg/kg) and cytotoxic drugs i.p. in doses described in Table 7.1. The drugs were reconstituted according to the

Table 7.1

Drugs assessed for synergy with PDT

Drug	Dose (mg/kg)
Methotrexate: David Bull Laboratories Pty. Ltd	0.1, 0.2
Doxorubicin HCl (Adriamycin): Farmitalia, Carlo Erbi S.p.A., Milan	0.5-4.0
Cyclophosphamide (Endoxan-Asta): Bristol	5, 10
5-Fluorouracil: Roche	12, 50
Vincristine sulphate (Oncovin): Eli Lilly (Aust)	0.001, 0.025
Thiotepa: Lederle	0.2, 0.4

manufacturer's instructions and diluted in saline immediately before use. The drug doses chosen represented the upper and lower ends of the therapeutic range recommended by the manufacturer. Twenty four hours later, the mice were given a second dose of cytotoxic drug i.p. and the tumours irradiated with 225J/sq cm from the incandescent lamp. TC_{50} and cutaneous photosensitivity were determined as described in Chapter 3. Each result represents the mean $\pm$ S.D. of two experiments. Differences between treatment groups were analysed by unpaired T tests.

Results

The tumours of mice treated with 30mg/kg HPD and 225J/sq cm light without cytotoxic drugs responded with a TC_{50} of 3.6 ± 0.47 days (three experiments). HPD or light alone did not affect the rate of tumour growth (Chapter 3).

The potentiation of PDT by Adriamycin administered at the time of HPD injection and before irradiation is illustrated in Fig. 7.1. The increase in TC_{50} from 3.6 to a maximum of 8 days was dependent on the dose of Adriamycin. A single dose of Adriamycin (3mg/kg) at the time of irradiation had a lesser effect, with a TC_{50} of 5 days. Control experiments of Adriamycin alone (3mg/kg), Adriamycin plus HPD, or Adriamycin plus light all had no visible effect on the rate of tumour growth. After administration of 3mg/kg Adriamycin, one mouse in each group usually died so higher doses of Adriamycin were not used.

The effect of five different cytotoxic drugs on the response of Lewis lung carcinoma to PDT is shown in Fig. 7.2. Slight increases in TC_{50} were observed with cyclophosphamide, vincristine and thiotepa but were not statistically significant

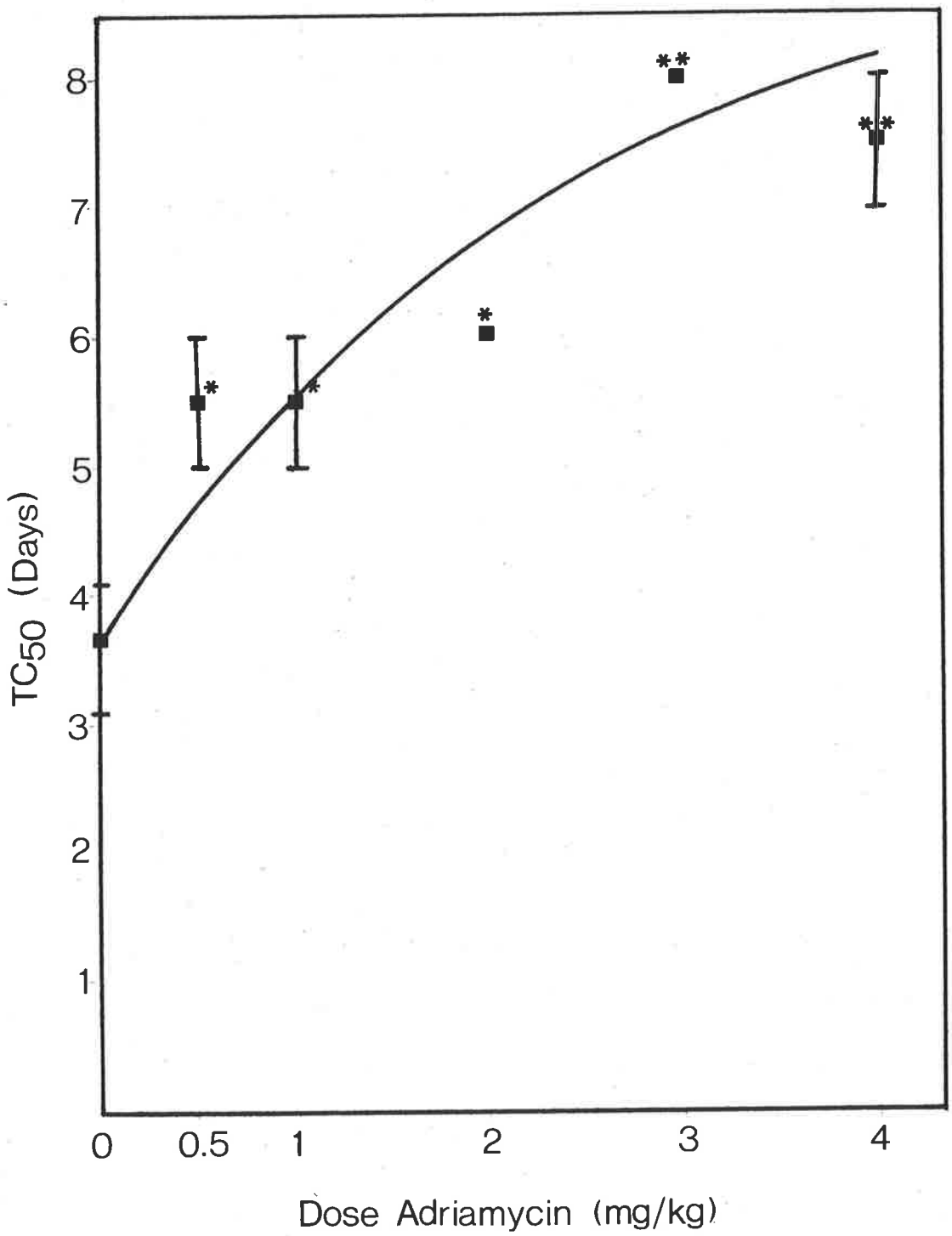


Fig. 7.1

Relationship between TC_{50} and dose of Adriamycin

Mice with Lewis lung carcinoma were given HPD (30mg/kg) and Adriamycin i.p. Twenty four hours later, mice were given a second dose of Adriamycin and the tumours irradiated.

* $p<0.02$

** $p<0.01$

Fig. 7.2

Influence of cytotoxic drugs on TC_{50}

Mice with Lewis lung carcinoma were given HPD (30mg/kg) and cytotoxic drugs i.p. Twenty four hours later, the mice were given a second dose of cytotoxic drug i.p. and the tumours irradiated.

A: No cytotoxic drugs.

B: cyclophosphamide, 5mg/kg

C: cyclophosphamide, 10mg/kg

D: methotrexate, 0.1mg/kg

E: methotrexate, 0.2mg/kg

F: 5-fluorouracil, 12mg/kg

G: 5-fluorouracil, 50mg/kg

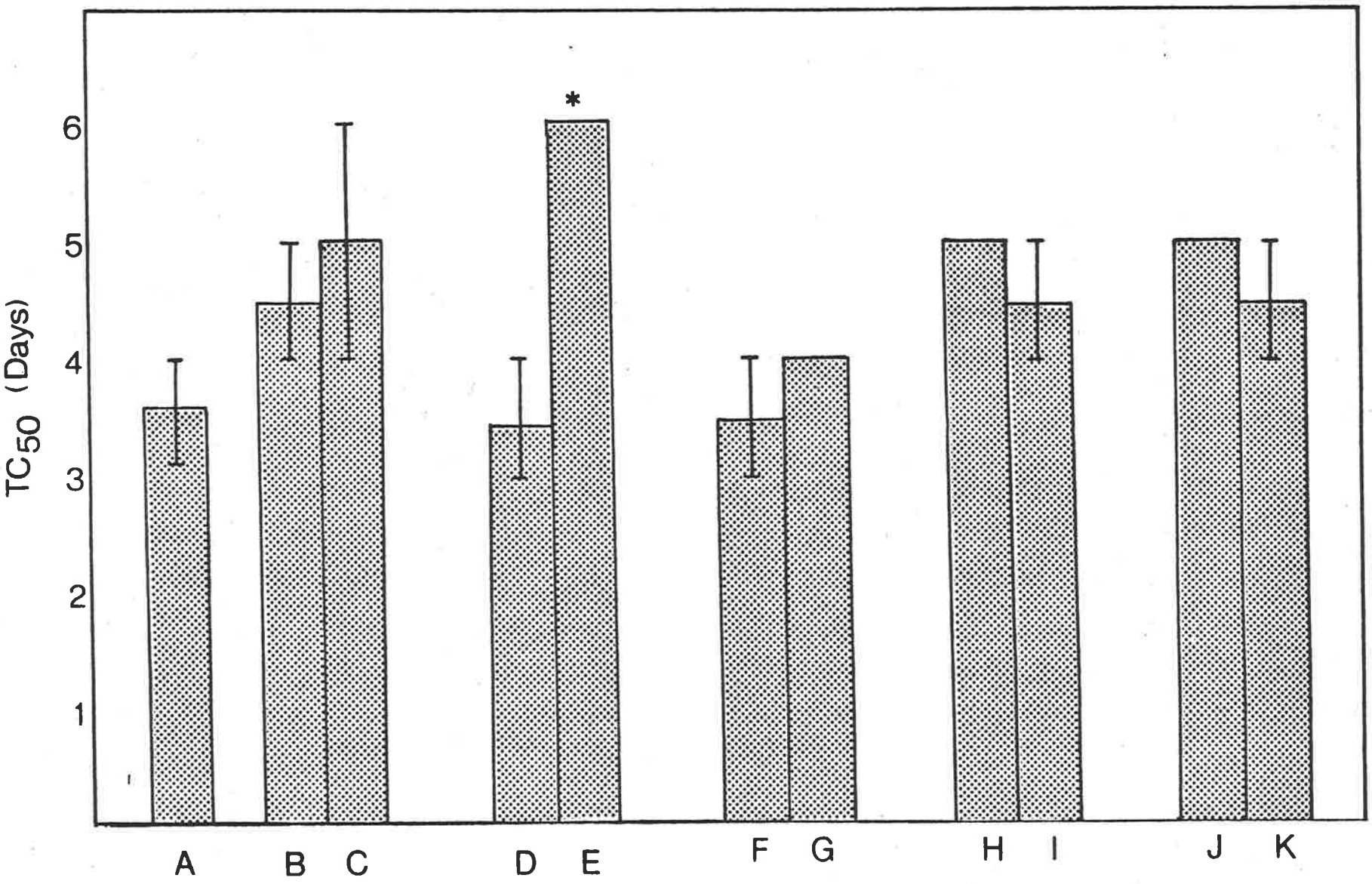
H: thiotepa, 0.2mg/kg

I: thiotepa, 0.4mg/kg

J: vincristine, 0.001mg/kg

K: vincristine, 0.025mg/kg

* $p<0.02$


TC_{50} (Days)
1
2
3
4
5
6
*
A
B
C
D
E
F
G
H
I
J
K

at $p<0.05$. 5-Fluorouracil had no effect at the doses tested. Methotrexate (0.2mg/kg) caused a two-fold increase in TC_{50} from 3.6 to 6 days, significant at the $p<0.02$ level. Drug alone, drug plus HPD or drug plus light all had no visible effect on tumour growth. At the higher drug doses, it was common for one mouse in each group to die.

The effect of cytotoxic drugs on cutaneous photosensitivity was also examined. There was considerable erythema and oedema in the treated footpad of most mice 24h after treatment with HPD and light only. Problems with reproducibility did not allow detection of a significant difference between this group and those also treated with cytotoxic drugs. Cytotoxic drugs did not appear to inhibit cutaneous photosensitivity.

(c). Influence of cytotoxic drugs on uptake of HPD

Methods

Uptake of HPD was assessed by fluorescence of frozen sections as described in Chapter 3. Mice with Lewis lung carcinoma were given HPD (30mg/kg) plus cytotoxic drugs i.p. at the higher doses shown in Table 7.1. Twenty four hours later, the mice were killed, the tumours frozen and sections examined for intensity of fluorescence.

Results

Fluorescence was more intense in tumours from mice receiving HPD plus cytotoxic drugs than in control tumours from mice receiving HPD only. The most intense fluorescence was seen following administration of Adriamycin or methotrexate, the drugs that resulted in the greatest potentiation of PDT. There was no increase in fluorescence after concurrent injection of

5-fluorouracil. This drug did not alter the TC_{50}.

(d). Effect of administration of Adriamycin after PDT

Methods

Mice were given HPD (30mg/kg i.p.) followed 24h later by 225J/sq cm light to the tumours. Twenty four and forty eight hours after irradiation, Adriamycin (3mg/kg i.p.) was administered and TC_{50} determined.

Results

Administration of Adriamycin after PDT resulted in a TC_{50} of 5 days in two separate experiments. This prolongation was not statistically significant at $p<0.05$ but suggests a slight potentiation of the photodynamic effect. Thus Adriamycin administered before PDT was much more effective in prolonging the duration of tumour control.

(e). Effect of Adriamycin on the photoactivity of HPD in vitro

Methods

The influence of Adriamycin on the photocytotoxic activity of HPD *in vitro* was examined using the ^{51}Cr release assay described in Chapter 2. ^{51}Cr-labelled Raji cells ($10X10^6$/ml in RPMI 1640) were incubated for 1h at 37^{o} with HPD (25ug/ml) and Adriamycin (0-200ug/ml). The cells were washed once, resuspended in PBS, irradiated for 0-20min and percentage ^{51}Cr determined immediately after irradiation as previously described. In some experiments, Raji cells were incubated with HPD, resuspended in PBS containing 0-20ug/ml Adriamycin and immediately irradiated. Background percentage ^{51}Cr release, determined from cells incubated with HPD or HPD plus Adriamycin but not exposed to light, was subtracted from all experimental

values. Adriamycin alone or Adriamycin plus light without HPD did not cause any ^{51}Cr release above the background.

Uptake of HPD in Raji cells was assessed by fluorescence as described in Chapter 2 after incubating the cells for 1h with HPD (25ug/ml) and graded doses of Adriamycin. Alternatively, Raji cells were incubated with HPD (25ug/ml), washed and resuspended in 0-20ug/ml Adriamycin. Cellular fluorescence was examined at once and again after 30min.

Results

The influence of Adriamycin on ^{51}Cr release from HPD-sensitized Raji cells is shown in Fig. 7.3. In the absence of Adriamycin, there was a linear increase in ^{51}Cr release with increasing irradiation time until maximum ^{51}Cr release (65-75%) was reached. Adriamycin caused a dose-dependent inhibition of ^{51}Cr release. There was a linear relationship between dose of Adriamycin and inhibition of ^{51}Cr release (Fig. 7.4).

^{51}Cr release was also inhibited by suspending HPD-sensitized Raji cells in graded doses of Adriamycin in PBS and then irradiating the cells (Fig. 7.5). The relationship between dose of Adriamycin and ^{51}Cr release is illustrated in Fig. 7.6.

The influence of Adriamycin on the uptake of HPD by Raji cells was assessed by fluorescence. Red HPD fluorescence decreased with increasing doses of Adriamycin and was absent in the cells incubated in 200ug/ml Adriamycin. The orange-yellow fluorescence of Adriamycin made detection of the red HPD fluorescence difficult. The effect of Adriamycin on the fluorescence of cells pretreated with HPD was also examined. After 30min in Adriamycin (10 or 20ug/ml), HPD fluorescence was greatly diminished, suggesting Adriamycin may cause efflux of

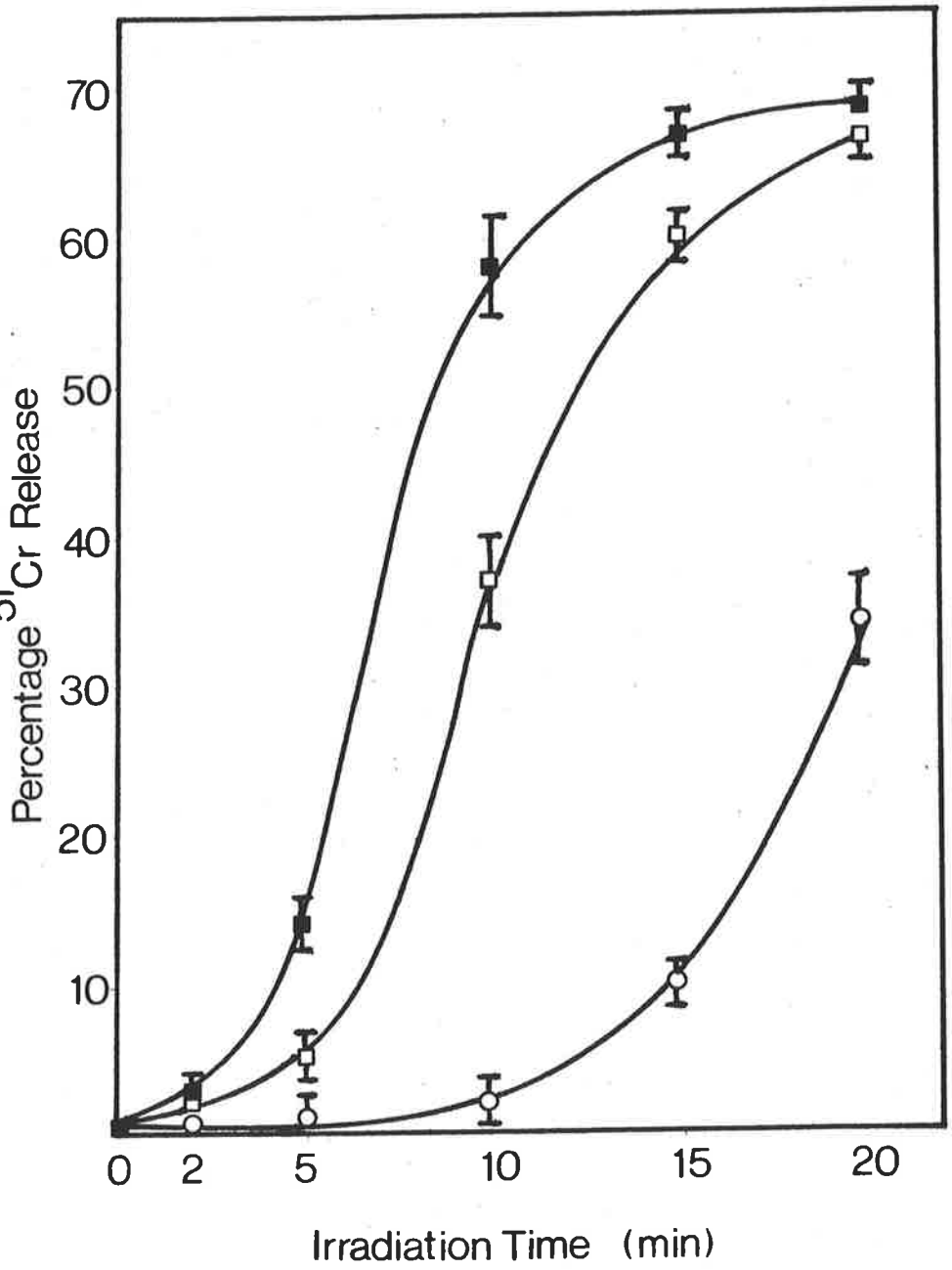


Fig 7.3

Influence of Adriamycin on ^{51}Cr release from HPD sensitized Raji cells

^{51}Cr-labelled Raji cells were incubated for 1h with HPD (25ug/ml) and Adriamycin (0-200ug/ml) and irradiated.

■ HPD (25ug/ml)

□ HPD (25ug/ml) plus Adriamycin (20ug/ml)

○ HPD (25ug/ml) plus Adriamycin (200ug/ml)

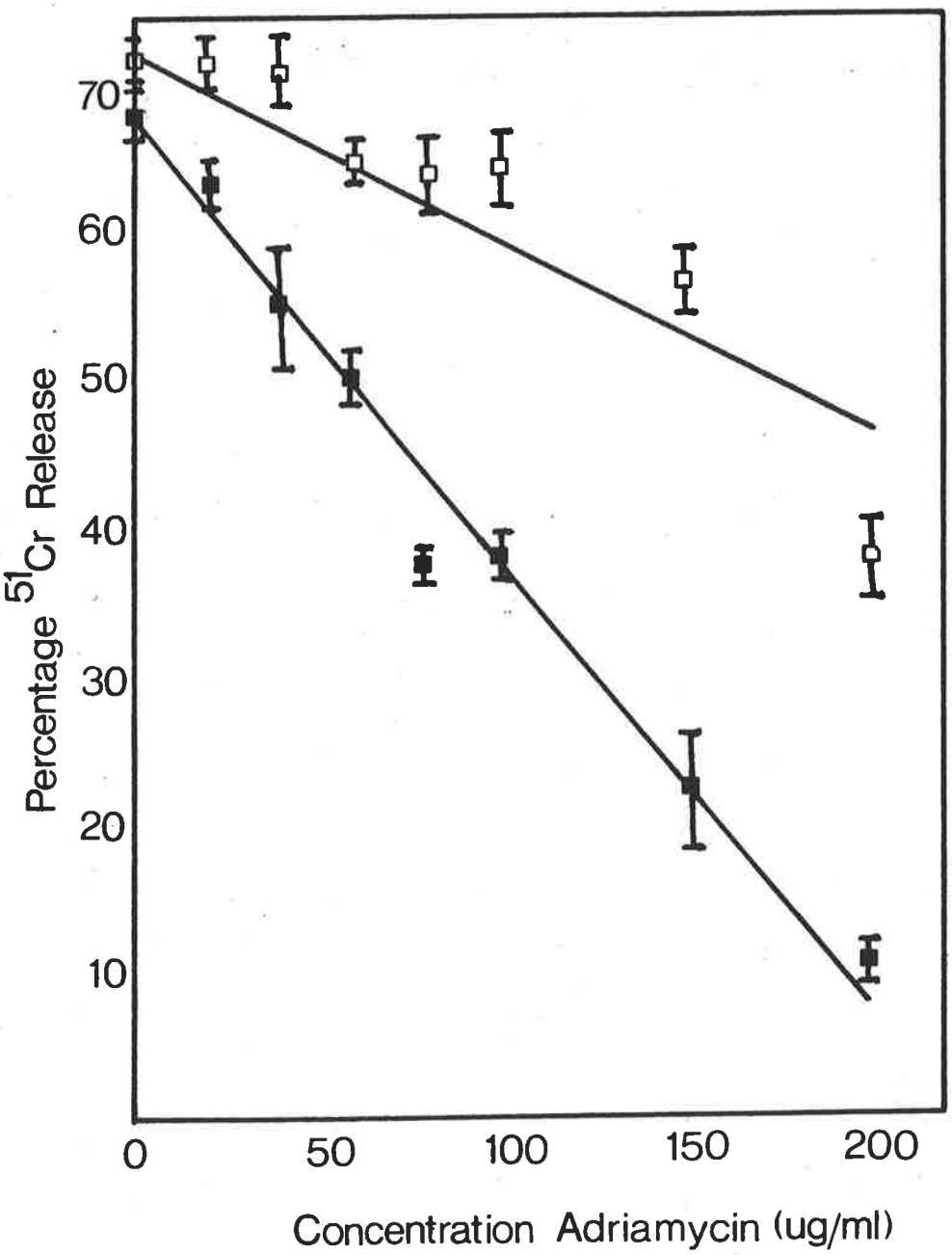


Fig. 7.4

Relationship between percentage ^{51}Cr release and concentration of Adriamycin

Cells were incubated in HPD plus Adriamycin, then irradiated. The data from Fig. 7.3 was used to plot percentage ^{51}Cr release against Adriamycin concentration.

□ 15min irradiation

■ 20min irradiation.

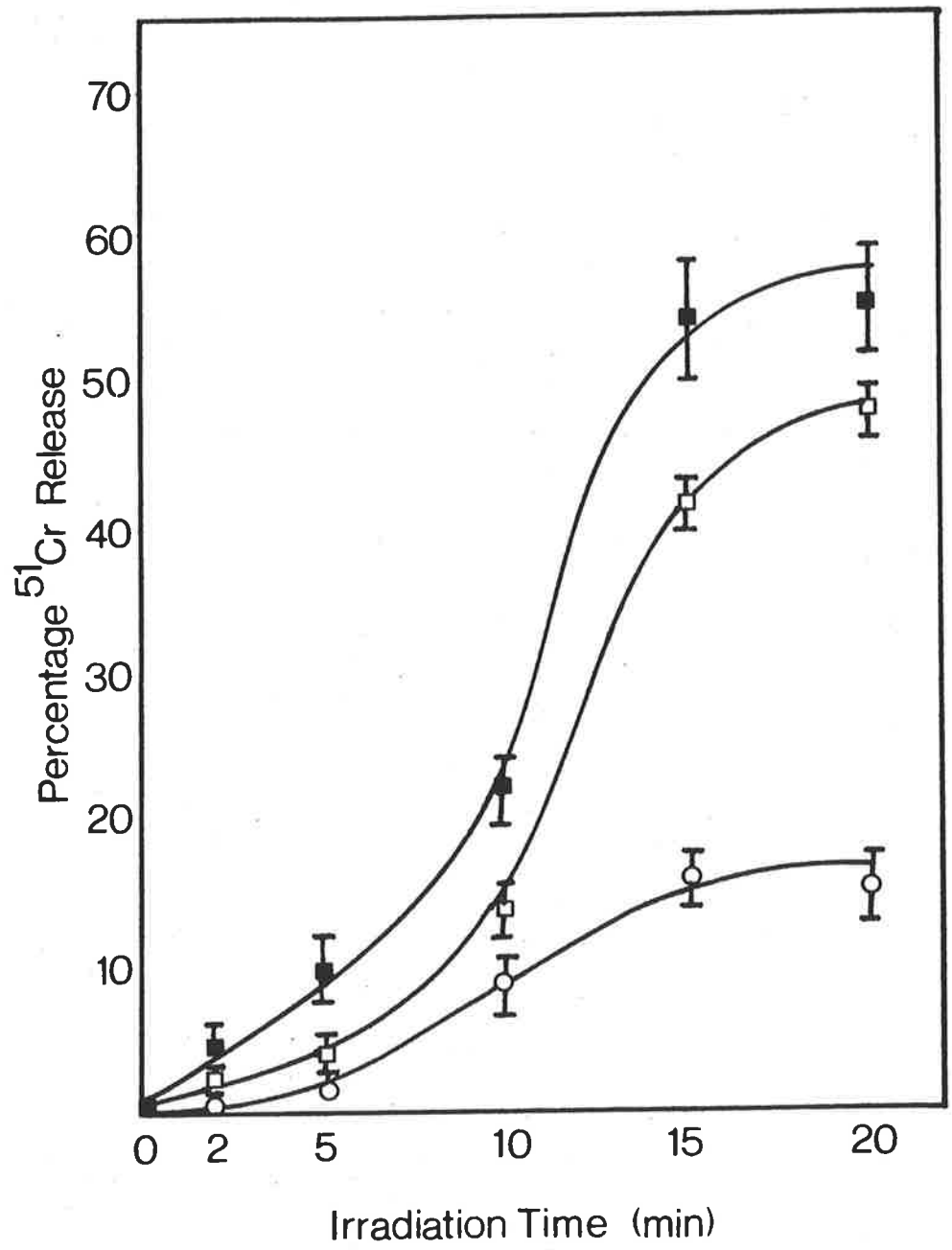


Fig. 7.5

Irradiation of HPD sensitized Raji cells

in the presence of Adriamycin

^{51}Cr-labelled Raji cells were incubated in HPD (25ug/ml) for 1h, resuspended in graded doses of Adriamycin in PBS and irradiated.

■ No Adriamycin.

□ Adriamycin (1ug/ml)

○ Adriamycin (10ug/ml)

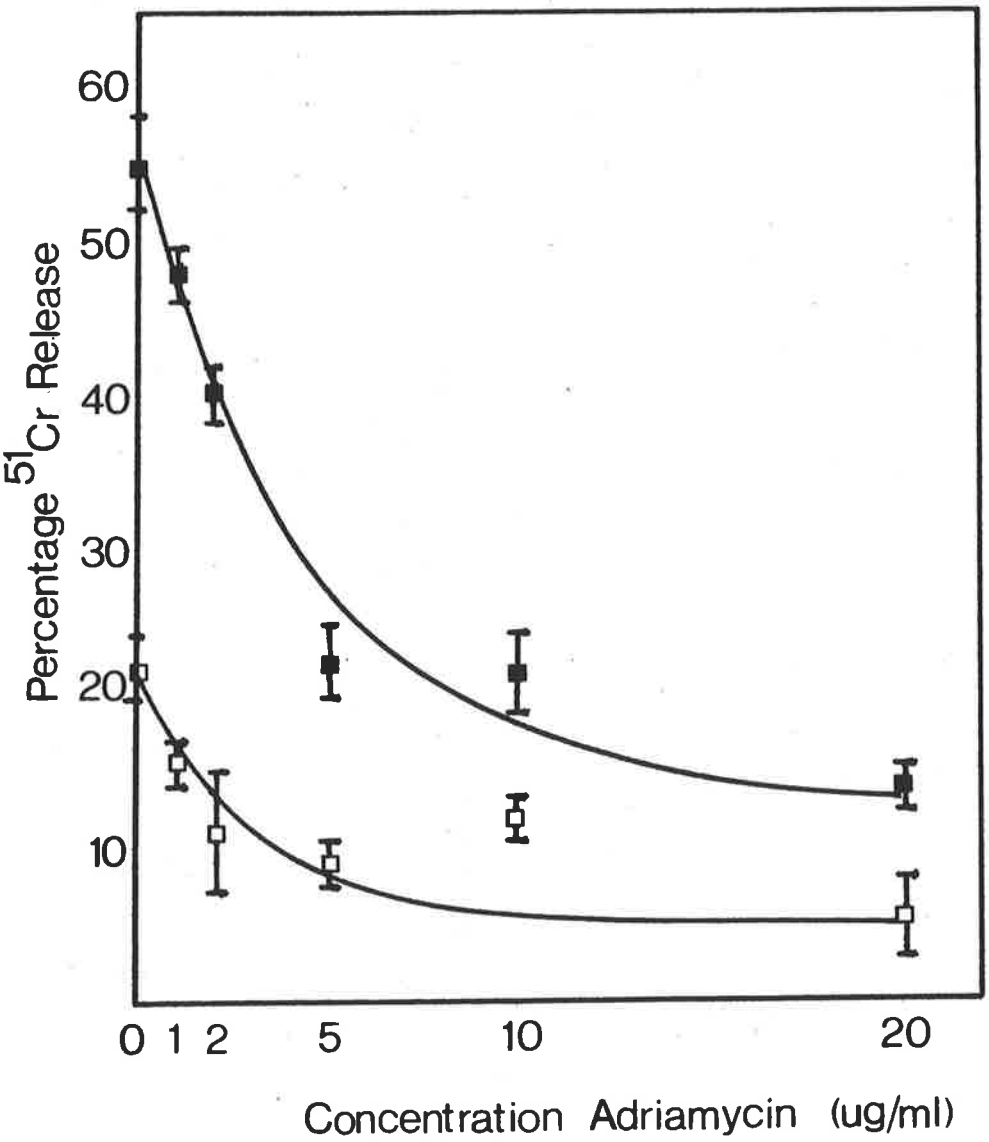


Fig. 7.6

Relationship between percentage ^{51}Cr release and concentration of Adriamycin present during irradiation

Cells were incubated in HPD (25ug/ml) for 1h, resuspended in graded doses of Adriamycin and irradiated. The data from Fig. 7.5 was used to plot percentage ^{51}Cr release against Adriamycin concentration.

■ 10min irradiation.

□ 15min irradiation.

HPD from the cells.

(f). Discussion

There have been few reports of interactions between PDT and cytotoxic drugs used for cancer chemotherapy. Gillio and Cortese [1985] demonstrated increased mortality when mice given Adriamycin in addition to HPD were irradiated to the whole body. Tumour response was not examined. Dougherty [1984b] reported severe reactions to PDT in patients treated with Adriamycin and then given PDT to cutaneous tumours. In the above experiments, Adriamycin caused an increase in the efficacy of photodynamic destruction of tumours. Creekmore and Zaharko [1983] also showed that actinomycin D or L-phenylalanine mustard were synergistic with HP and light in the inactivation of L1210 cells.

In the present study, the transplantable tumour model described in Chapter 3 was used to investigate interactions between PDT and cytotoxic drugs. The drugs chosen represented some of the major classes of compound in common clinical use. Doses used corresponded to the therapeutic range for human tumours and were approaching toxic levels for mice.

The Lewis lung carcinoma appears to be relatively resistant to chemotherapy. Several studies have examined the sensitivity of Lewis lung carcinoma to cyclophosphamide. Karrer et al. [1967] reported that 300mg/kg cyclophosphamide every fifth day did not increase survival in mice with Lewis lung carcinoma. However Steel and Adams [1975] found that Lewis lung carcinoma cells were sensitive to cyclophosphamide (20-300mg/kg) as judged by *in vitro* colony formation. Mayo [1972] reported that cyclophosphamide cured early tumours and was

effective as adjuvant chemotherapy after surgery, but did not cause regression of established tumours. Stephens et al. [1981] reported that Lewis lung carcinoma was sensitive to 40-120mg/kg cyclophosphamide, 100-300mg/kg 5-fluorouracil and responded slightly to 0.5-2.0mg/kg vincristine. Lewis lung carcinoma was sensitive to Adriamycin at high doses (5-20mg/kg), with delays in tumour growth and reduction in size [Martini et al., 1977; Donelli et al., 1979; Broggini et al., 1983]. For the present study, drug doses were chosen that would not be effective alone, so that synergistic effects between cytotoxic drugs and PDT would be detected.

Adriamycin and methotrexate were the most effective drugs in potentiating the tumour response to PDT. Both these drugs were associated with increased intensity of HPD fluorescence in the tumour, suggesting that the potentiation of PDT by drugs results from greater uptake or retention of HPD in the tumour. The role of the vasculature in PDT was discussed in Chapter 1. Cytotoxic drugs could act by damaging the vascular system to allow greater HPD uptake. After irradiation, the drugs could also increase the destruction or inhibit repair of capillaries in PDT-treated tumours. Inhibition of repair from sublethal damage to tumour cells caused by PDT is also a potential mechanism by which cytotoxic drugs may enhance PDT. Adriamycin [Donelli et al., 1979; Broggini et al., 1983; Dorr and Alberts, 1982] and HPD [Berns et al., 1982] both accumulate in the mitochondria so cumulative damage to the cells could be expected. Similarly HPD [Lee See et al., 1984; Weishaupt et al., 1976] and Adriamycin [Dorr and Alberts, 1982; Salazar and Cohen, 1984] both have been postulated to inactivate cells by radical-

mediated mechanisms so additive effects may be expected. The other drugs tested did not show any synergy with PDT although both cyclophosphamide and 5-fluorouracil [Mayo, 1972; Stephens et al., 1981] affect the growth of Lewis lung carcinoma. Higher drug doses could not be tested as toxicity was observed at the doses used.

In contrast to the synergy observed between Adriamycin and PDT *in vivo*, Adriamycin caused marked inhibition of ^{51}Cr release from HPD-sensitized Raji cells *in vitro*. Adriamycin inhibited uptake of HPD and also appeared to cause efflux of HPD from the cells. Adriamycin may inhibit uptake of HPD by its effects on the plasma membrane. It increases hydrophilic glycoproteins in the membrane [Kessel, 1979]. Since the most phototoxic component of HPD is the most hydrophobic (Chapters 4 and 5), uptake of the active component of HPD may be expected to be reduced. Adriamycin is known to modify other cell surface molecules, for example the Con A receptor [Murphree et al., 1976] so alterations in HPD uptake and efflux could be expected. The inhibition *in vitro* of PDT by Adriamycin does not have any therapeutic significance since it does not occur *in vivo*.

Reduction of cutaneous photosensitivity by cytotoxic drugs was not observed within the limitations of the assay. The footpads of all mice responded with marked erythema and oedema after irradiation.

In conclusion, the interactions between PDT and cytotoxic drugs merit further investigation. Synergy was detected between PDT and Adriamycin or methotrexate. Thus the combined use of both therapies, while not having the desired effects alone, may lead to tumour control with tolerable side effects.

CHAPTER 8

MANIPULATION OF PHOTODYNAMIC ACTIVITY BY GLUCOCORTICOIDS

(a). Introduction

The previous chapter considered the modification of the response of tumours to PDT by concurrent administration of cytotoxic drugs. Since glucocorticoids are also used frequently in the treatment of cancer, it was decided to investigate any interactions between PDT and glucocorticoids. The antiinflammatory action of glucocorticoids may lead to altered tumour destruction or may affect the rate of recurrence of tumours. The action of glucocorticoids on the vascular system may also affect uptake of HPD and hence efficacy of PDT.

(b). Effect of administration of glucocorticoids before PDT

Methods

The transplantable tumour model in mice described in Chapter 3 was used to assess the influence of glucocorticoids on PDT. Mice with Lewis lung carcinoma or B16 melanoma were given HPD (30mg/kg i.p.) and methylprednisolone acetate (Depo-medrol, Upjohn) (0.6-3.0mg/kg i.p.) or hydrocortisone sodium succinate (Efcortelan, Glaxo) (15mg/kg i.p.). Equivalent doses of the two glucocorticoids were chosen [Haynes and Murad, 1980]. Twenty four hours later, a second dose of glucocorticoid was given and the tumours irradiated with 225J/sq cm for Lewis lung carcinoma and 110J/sq cm for B16 melanoma. TC_{50} was determined as previously described. All results are expressed as the mean $\pm$ S.D. of two experiments. Differences between treatments were analysed by unpaired T-tests.

Results

Mice with Lewis lung carcinoma treated with HPD (30mg/kg i.p.) and 225J/sq cm light without glucocorticoids responded with a TC_{50} of 3.6±0.47 days (three experiments). Mice with B16 melanoma treated with HPD (30mg/kg i.p.) and 110J/sq cm light without glucocorticoids responded with a TC_{50} of 5 days (two experiments).

Hydrocortisone sodium succinate (15mg/kg) administered with HPD and before irradiation to mice with Lewis lung carcinoma did not significantly alter the TC_{50}, which remained at 3 days. Similarly, hydrocortisone sodium succinate did not alter the response of B16 melanoma to PDT, with the TC_{50} remaining at 5 days. Hydrocortisone sodium succinate alone, hydrocortisone sodium succinate plus HPD without light, or hydrocortisone sodium succinate plus light had no visible effect on the growth of either tumour. When hydrocortisone sodium succinate was administered at the time of transplantation of tumours, the time for appearance of palpable tumour was still 7-10 days for both tumours.

Methylprednisolone acetate administered with HPD and before irradiation had a marked influence on the response of Lewis lung carcinoma to PDT (Fig. 8.1). The TC_{50} increased to 5 days after administration of a low dose of glucocorticoid (0.6mg/kg). There was then a dose-dependent inhibition of the tumour response until, with 3mg/kg, only 50% of the tumours were impalpable 24h after treatment (TC_{50} of one day). There was no reduction in size of tumours in mice treated with methylprednisolone acetate alone, methylprednisolone acetate plus HPD, or methylprednisolone acetate plus light. When methylprednisolone acetate (3.0mg/kg) was administered at the

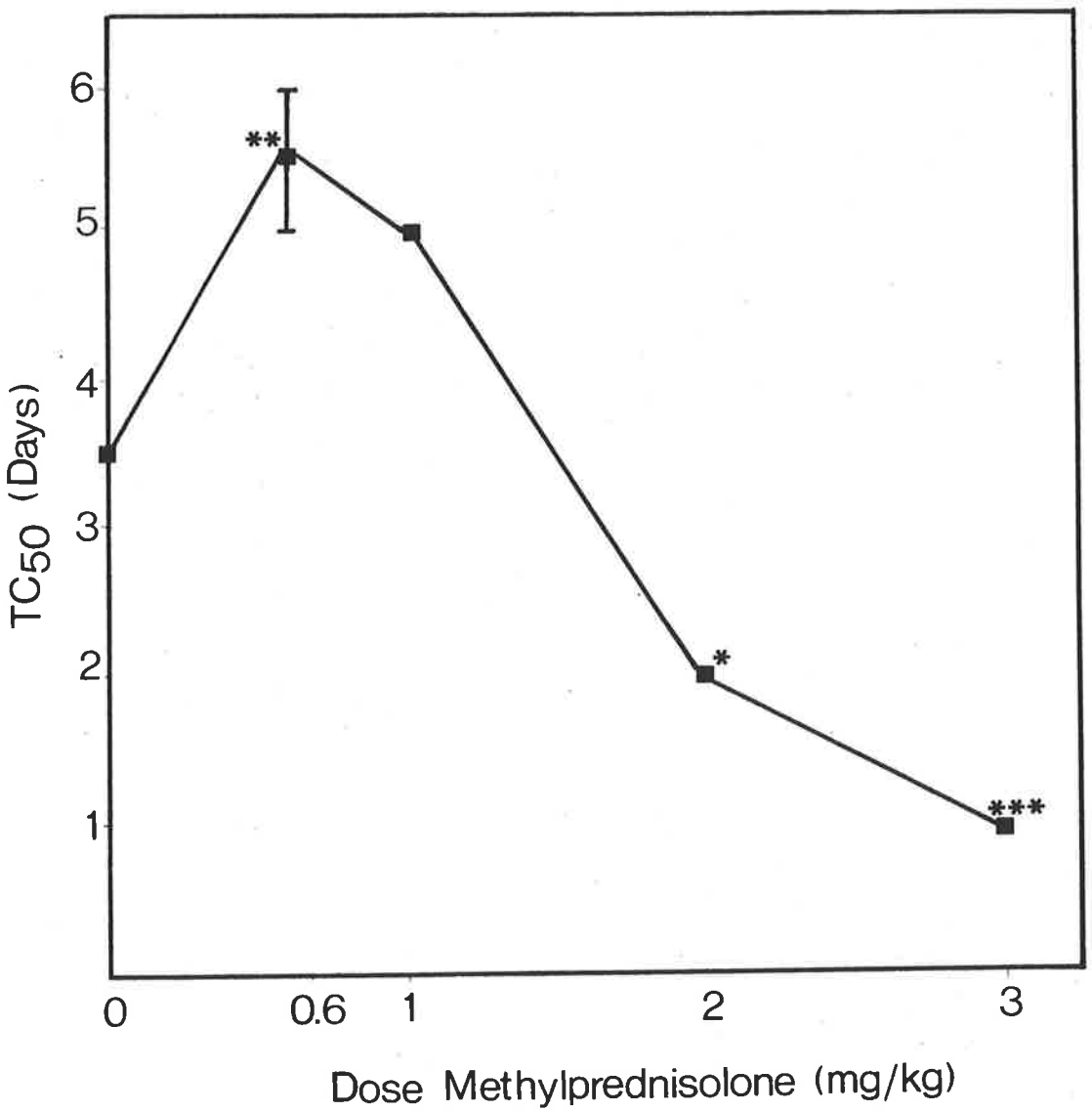


Fig. 8.1

Relationship between TC_{50} and dose of methylprednisolone acetate

Mice with Lewis lung carcinoma were given HPD (30mg/kg i.p.) plus methylprednisolone acetate i.p. Twenty four hours later, a second dose of methylprednisolone acetate was given and the tumours irradiated.

* $p<0.05$

** $p<0.02$

*** $p<0.01$

time of transplant with Lewis lung carcinoma, tumours still became palpable 7-10 days later, indicating that methylprednisolone acetate had no effect on the rate of tumour growth.

There was also a marked inhibition of the response of B16 melanoma to PDT after administration of methylprednisolone acetate (3mg/kg) concurrently with HPD and before irradiation. Most tumours were still palpable 24h after irradiation. Control experiments, as described above, showed that methylprednisolone acetate alone had no effect on the rate of tumour growth and did not cause regression of the tumours.

(c). Effect of administration of glucocorticoids after PDT

Methods

Mice with Lewis lung carcinoma were given HPD (30mg/kg i.p). followed 24h later by irradiation of the tumours with 225J/sq cm light. Mice with B16 melanoma were given HPD (30mg/kg i.p.) followed 24h later by irradiation of the tumours with 110J/sq cm light. Twenty four and forty eight hours after irradiation, the mice were given glucocorticoids i.p. in doses described in Fig. 8.2. TC_{50} was determined as previously described.

Results

All the glucocorticoids caused marked potentiation of the response of Lewis lung carcinoma to PDT (Fig. 8.2). Hydrocortisone sodium succinate was the most effective. Methylprednisolone acetate was more effective than methylprednisolone sodium succinate (Solu-medrol, Upjohn), suggesting that a longer lasting form of glucocorticoid may be more effective.

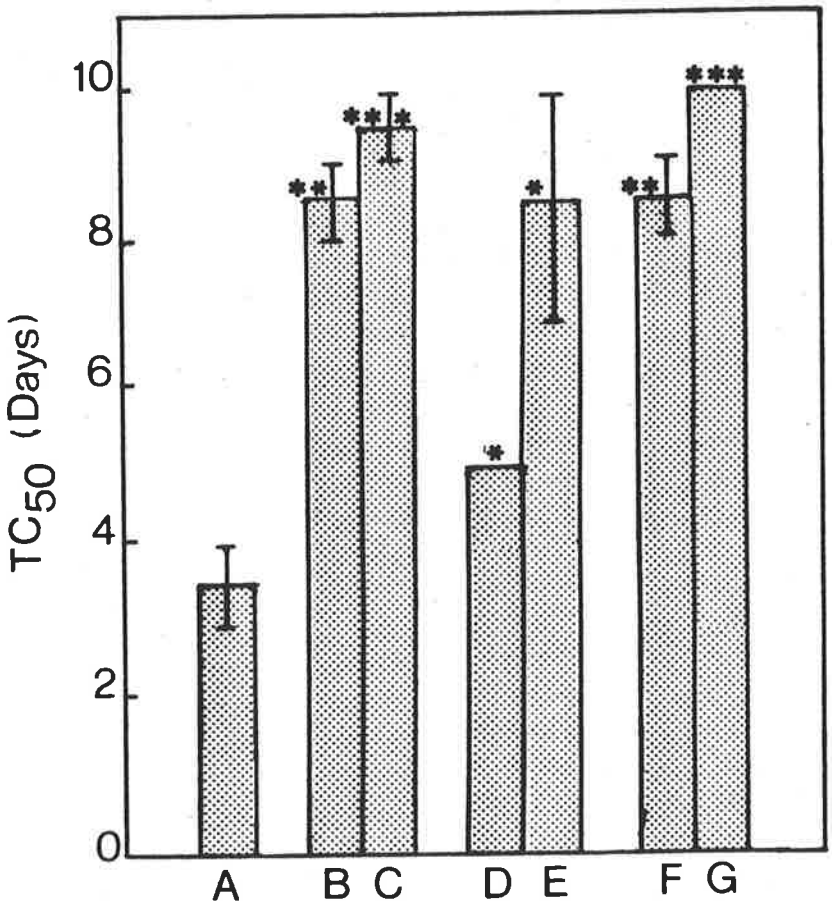


Fig. 8.2

Effect of administration of glucocorticoids after PDT

Mice with Lewis lung carcinoma were given HPD (30mg/kg i.p.) and the tumours irradiated 24h later. Glucocorticoids were administered i.p. 24 and 48h after irradiation.

A: No glucocorticoid.

B: methylprednisolone acetate, 0.6mg/kg

C: methylprednisolone acetate, 3.0mg/kg

D: methylprednisolone sodium succinate, 0.6mg/kg

E: methylprednisolone sodium succinate, 3.0mg/kg

F: hydrocortisone sodium succinate, 3.0mg/kg

G: hydrocortisone sodium succinate, 15.0mg/kg

* $p<0.05$

** $p<0.01$

*** $p<0.001$

Recurrence of B16 melanoma was also inhibited by administration of hydrocortisone sodium succinate 24 and 48h after irradiation. TC_{50} was increased from 5 days to 7.5±0.5 days ($p<0.01$) and to 8.5±0.5 days ($p<0.001$) after 3 or 15mg/kg glucocorticoid respectively. The effect was not as marked as that observed with Lewis lung carcinoma (Fig. 8.2).

(d). Influence of glucocorticoids on skin photosensitivity

Methods

Mice with Lewis lung carcinoma were given HPD (30mg/kg i.p.) and methylprednisolone acetate (3mg/kg i.p). Twenty four hours later, the left footpad was irradiated with 225J/sq cm light and percentage increase in footpad thickness determined as described in Chapter 3.

Results

Administration of methylprednisolone acetate had no effect on skin photosensitivity. The mean percentage increase in footpad thickness was 36±20% in mice treated with HPD only and 41±14% in mice treated with HPD plus methylprednisolone acetate.

(e). Effect of glucocorticoids on uptake of HPD

Methods

Mice with Lewis lung carcinoma were given HPD (30mg/kg) and methylprednisolone acetate (0.6-3.0mg/kg) or hydrocortisone sodium succinate (15mg/kg). Mice with B16 melanoma were given HPD (30mg/kg) and methylprednisolone acetate (3.0mg/kg) or hydrocortisone sodium succinate (15mg/kg). Twenty four hours later, the mice were killed and fluorescence of frozen sections examined as described in Chapter 3.

Results

The intensity of fluorescence in Lewis lung carcinoma progressively decreased when increasing doses of methylprednisolone acetate were administered concurrently with HPD. Fluorescence was very faint in tumours after high doses of methylprednisolone acetate (3.0mg/kg). Hydrocortisone sodium succinate (15 mg/kg) administered concurrently with HPD did not alter the intensity of fluorescence. The efficacy of PDT corresponded with the intensity of fluorescence in the tumours.

Methylprednisolone acetate (3.0mg/kg) also caused a reduction in intensity of fluorescence in B16 melanoma while hydrocortisone sodium succinate (15mg/kg) caused a slight increase in intensity of fluorescence.

(f). Influence of glucocorticoids on phototoxicity in vitro

Method

Lewis lung carcinoma cells were grown in vitro and harvested as described in Chapter 3. The cells ($10X10^6$/ml) were incubated for 1h in RPMI 1640/10%FCS with HPD (0-50ug/ml). The cells were washed and resuspended at $0.2X10^6$/ml in RPMI 1640/10%FCS and irradiated for 0-20min as described in Chapter 2. Quadruplicates (100ul) of the irradiated cells were plated out in in flat bottomed microculture plates (Linbro) and 100ul of hydrocortisone sodium succinate (0-1000ug/ml) in RPMI 1640/10%FCS added to each well. The plates were incubated for 21h at 37^o in $5\%CO_2$. ^{3}H-thymidine (1uCi) was added 18h before harvesting the cultures. Thirty minutes before harvesting, 50ul of 0.01% trypsin in PBS was added to each well. The cultures were harvested with a Skatron cell harvester and the filter discs counted as described in Chapter 2. Results were obtained

in counts per minute. ^{3}H-thymidine uptake by cells that were not treated with HPD or hydrocortisone sodium succinate or irradiated was taken as 100%. Percentage inhibition of ^{3}H-thymidine uptake was calculated and each point represents the mean ± S.D. of quadruplicates.

Results

The influence of HPD and light on ^{3}H-thymidine uptake by Lewis lung carcinoma cells is illustrated in Fig. 8.3. Inhibition of ^{3}H-thymidine uptake was dependent on the dose of both HPD and light. ^{3}H-thymidine uptake by Lewis lung carcinoma cells was also progressively inhibited by increasing doses of hydrocortisone sodium succinate (Fig. 8.4).

The effect of combining the two treatments is shown in Fig. 8.5. Doses of HPD and hydrocortisone sodium succinate were chosen to cause suboptimal inhibition of ^{3}H-thymidine uptake. Inhibition by HPD and glucocorticoid was additive rather than synergistic. There was light-dependent inhibition of ^{3}H-thymidine uptake when cells were cultured in hydrocortisone sodium succinate. This could be due to slight hyperthermia to the cells during irradiation causing increased susceptibility to glucocorticoid-induced inhibition of ^{3}H-thymidine uptake.

(g). Discussion

Glucocorticoids administered after PDT greatly potentiated the therapeutic effect by slowing the rate of recurrence of tumours. The most effective glucocorticoid was hydrocortisone sodium succinate. In contrast, a high dose of methylprednisolone acetate administered at the same time as HPD strongly inhibited the response to treatment. These effects were seen with both Lewis lung carcinoma and B16 melanoma. The

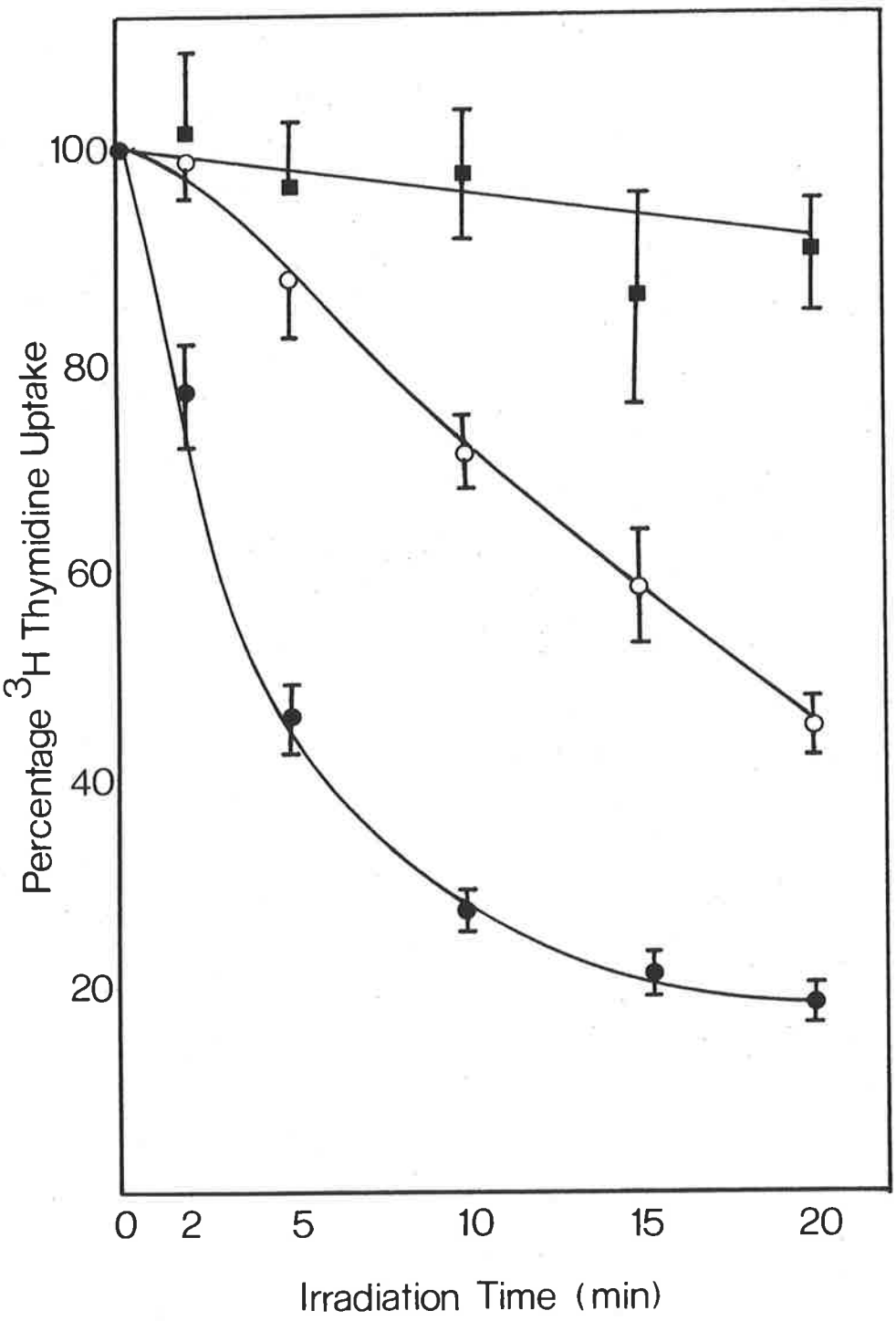


Fig. 8.3

Inhibition by HPD and light of ^{3}H-thymidine uptake by Lewis lung carcinoma cells in vitro

Lewis lung carcinoma cells were incubated with HPD, irradiated and cultured for 21h at 37°. ^{3}H-thymidine was added 18h before the cultures were harvested.

■ No HPD

○ HPD (20ug/ml)

● HPD (50ug/ml)

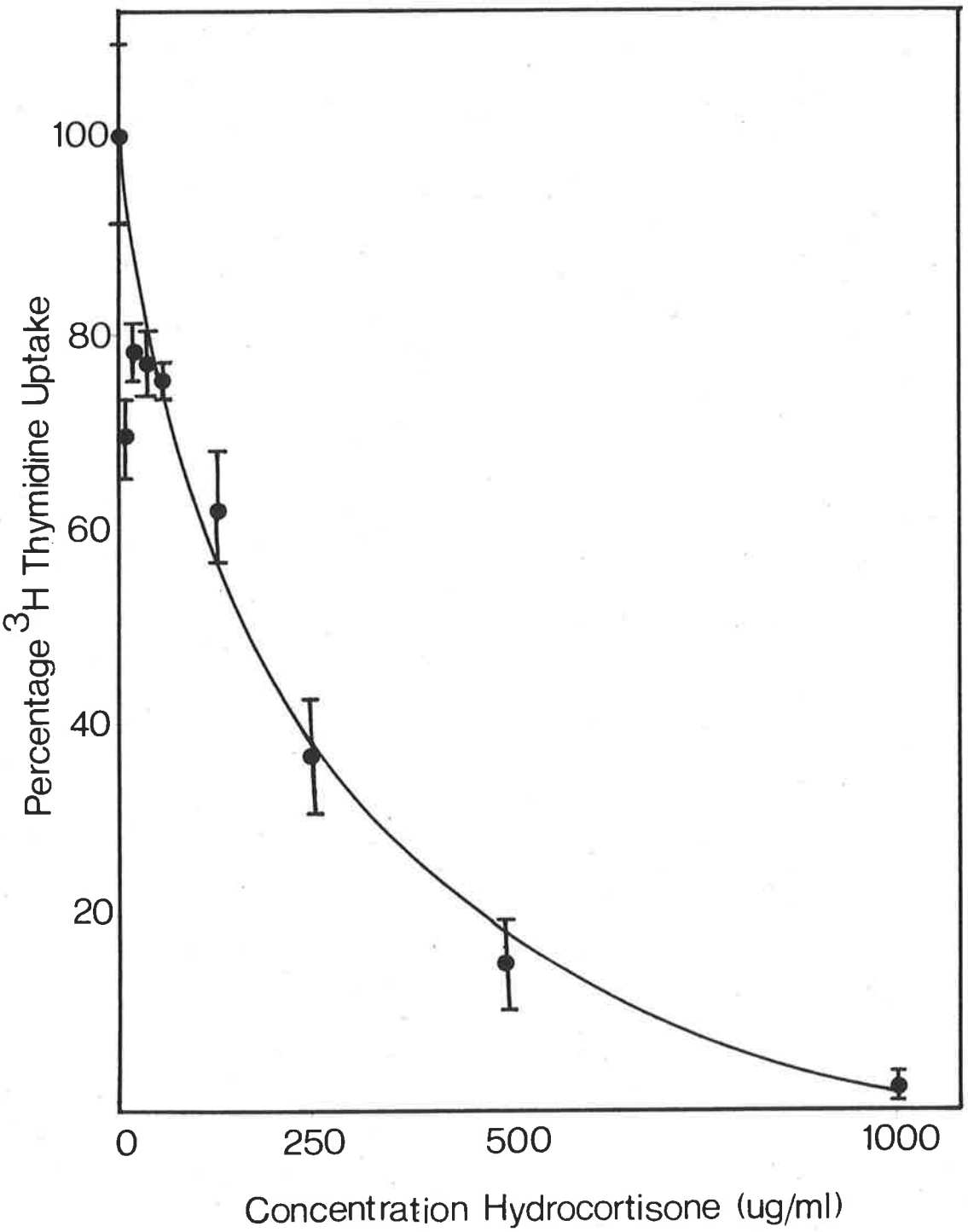


Fig. 8.4

Inhibition by hydrocortisone of ^{3}H-thymidine uptake by Lewis lung carcinoma cells in vitro.

Lewis lung carcinoma cells were cultured for 21h in graded doses of hydrocortisone sodium succinate. ^{3}H-thymidine was added to the cultures 18h before harvesting.

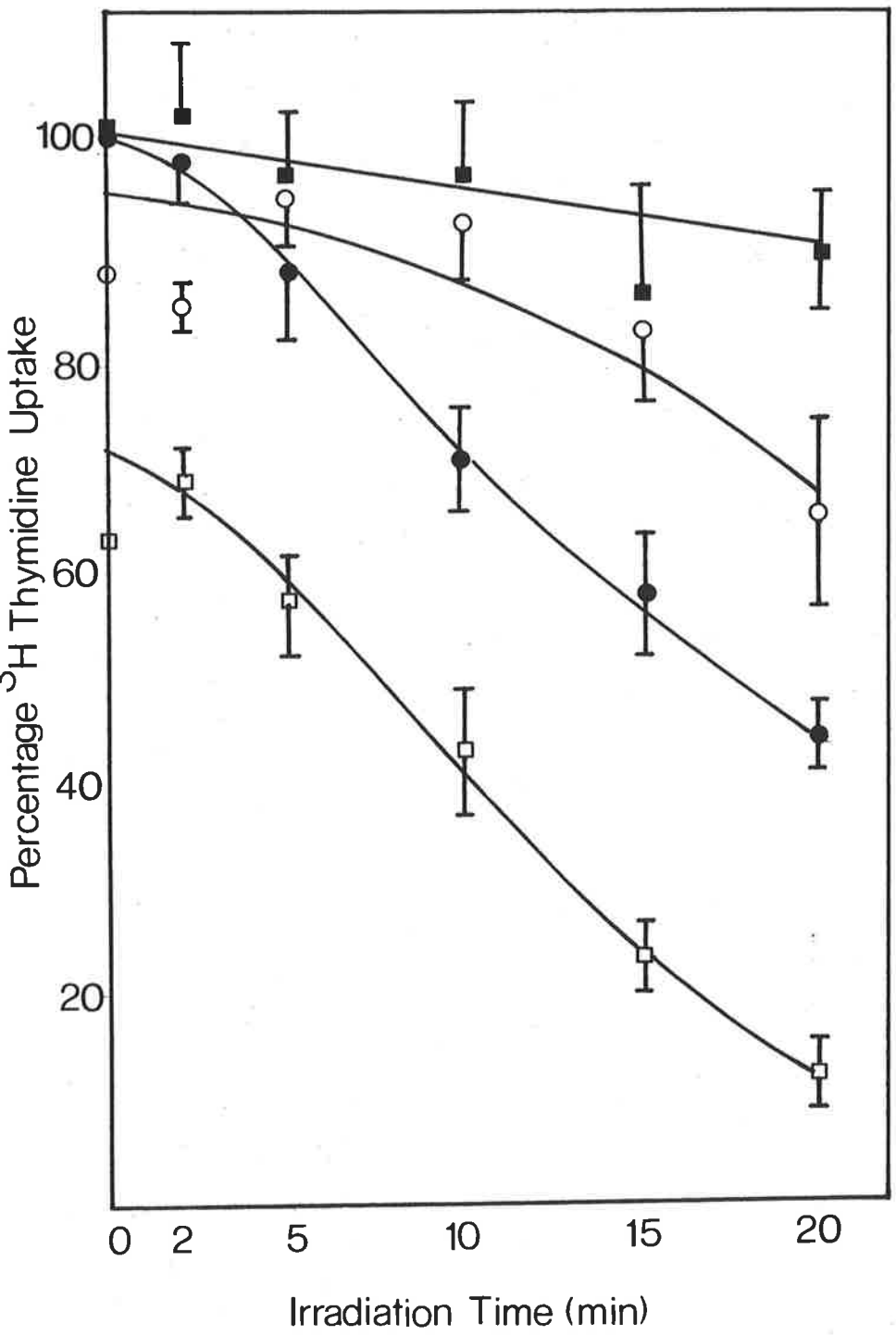


Fig. 8.5

Inhibition by PDT plus hydrocortisone of ^{3}H-thymidine uptake by Lewis lung carcinoma cells in vitro

Lewis lung carcinoma cells were incubated in HPD, irradiated and cultured for 2lh in hydrocortisone sodium succinate. ^{3}H-thymidine was added 18h before harvesting.

- ● HPD (25ug/ml)
- ○ Hydrocortisone sodium succinate (100ug/ml)
- □ HPD plus hydrocortisone sodium succinate (100ug/ml)
- ■ Control

inhibition of PDT by methylprednisolone acetate was dose-dependent, with the lowest dose of methylprednisolone acetate (0.6mg/kg) resulting in a prolonged TC_{50}.

Intensity of fluorescence in the tumours decreased with increasing doses of methylprednisolone acetate, implying that glucocorticoid inhibited the uptake of HPD. Reduced therapeutic response was associated with reduced intensity of fluorescence. Hydrocortisone sodium succinate (15mg/kg) did not alter either the fluorescence intensity or the response of the tumours to PDT.

Both the inhibition of uptake of HPD into tumours by concurrent administration of glucocorticoid and the inhibition of tumour recurrence by administration of glucocorticoid after an effective PDT treatment need to be explained.

Localization of HPD is 5 times greater in the vascular stroma than in tumour cells [Bugelski et al., 1981]. Glucocorticoids may inhibit uptake of HPD by decreasing capillary permeability [Claman, 1975; Haynes and Murad, 1980]. Henderson et al. [1985] showed tumour cells are not killed immediately after irradiation but die at a rate similar to that occurring after vascular occlusion, implying the tumours die by infarct. Thus any effect of glucocorticoids to preserve the vasculature will protect against photodynamic damage.

Digestion of phagocytosed material may be depressed by hydrocortisone [Handin and Stossel, 1978]. Since a proportion of HPD may be taken up by phagocytosis [Moan and Christensen, 1980], glucocorticoids may depress uptake of HPD by this mechanism.

Glucocorticoids are transported through the body by binding to plasma proteins [Guyton, 1981] and porphyrins also

bind to albumin and haemopexin (discussed in Chapter 1). Competition between HPD and glucocorticoid for binding sites on albumin or other proteins may affect transport of HPD, with unpredictable effects on the uptake of HPD into tumours.

A primary event in porphyrin-induced photodynamic damage may be rupture of lysosomes with release of hydrolytic enzymes (discussed in Chapter 1). This process may be inhibited by glucocorticoids which stabilise lysosomal membranes [Weissmann, 1964; Guyton, 1981]. However lysosomal damage may not be a prerequisite for photodynamic damage (Chapter 1).

Administration of glucocorticoids after PDT may inhibit repair of photodynamic damage and division of tumour cells. Repair of the microvasculature may also be retarded by glucocorticoids. When Lewis lung carcinoma cells were grown in the presence of hydrocortisone, there was a dose dependent inhibition in the rate of ^{3}H-thymidine uptake, implying cell division was inhibited. However, this inhibition of growth was not evident in the transplantable mouse tumour, since the rate of tumour growth was not affected by concurrent administration of hydrocortisone sodium succinate at the time of transplantation. However local glucocorticoid concentration in the tumours of the mice may be much lower than that required to cause inhibition of growth in vitro. The division of Lewis lung carcinoma cells was also sensitive to treatment with HPD and light. When cells were treated in vitro with both HPD and light and hydrocortisone sodium succinate, the inhibition of ^{3}H-thymidine uptake was additive rather than synergistic.

Both the inhibitory effect of glucocorticoids on PDT and inhibition of tumour recurrence may have important implications

for the clinical application of PDT. It may be necessary to suspend glucocorticoid treatment for some time before PDT to obtain an adequate tumour response, or alternately use higher doses of HPD or light during treatment with glucocorticoids. Administration of glucocorticoids after an effective PDT treatment may reduce the likelihood of tumour recurrence. Unfortunately glucocorticoids had no detectable effect on the major side effect of skin photosensitivity.

CHAPTER 9

INFLUENCE OF VASOACTIVE DRUGS ON PHOTODYNAMIC THERAPY

(a). Introduction

The role of the tumour vasculature in the photodynamic destruction of tumours was discussed in Chapter 1. HPD accumulates and is preferentially retained in the vascular stroma [Bugelski et al., 1981]. Similarly, tumour necrosis appears to be a result of vascular damage.

By modifying the state of the tumour vasculature, vasoactive drugs could influence both the uptake of HPD and the efficacy of tumour destruction. Vasodilation, an increase in blood flow or an increase in capillary permeability could all be predicted to increase HPD uptake, similarly vasoconstriction may inhibit uptake. These influences could be clinically useful in improving the efficacy of PDT. Many patients may be concurrently treated with vasodilators so any interactions between the two therapies should be documented. The mechanism of action of PDT may also be investigated by examining the influence of vasoactive drugs on the destruction of tumours.

(b). Influence of verapamil on the efficacy of PDT

Methods

Mice with Lewis lung carcinoma were given HPD (30mg/kg i.p.) and verapamil (Isoptin, Knoll, A.G., 2.0 or 10.0mg/kg i.p). The lower dose of verapamil was double that previously reported to cause vasodilation of the blood vessels of rat tumours [Kaelin et al., 1982]. Twenty four hours later, tumours were irradiated with 225J/sq cm and TC_{50} determined as described in Chapter 3. In other experiments, verapamil

(2.0mg/kg i.v.) was administered either concurrently with HPD (i.p.) or immediately before irradiation. Alternatively, verapamil (2mg/kg i.p.) was administered 24 and 48h after irradiation.

Results

Verapamil (2 or 10mg/kg i.p.) administered with HPD potentiated the response of the tumours to PDT (Fig. 9.1). There was no relationship between TC_{50} and dose of verapamil, suggesting an optimum effect was reached with the lower dose. Verapamil (2mg/kg) administered intravenously was equally effective. Verapamil administered 24 and 48h after irradiation also increased the TC_{50} to 6 days. The mice given verapamil immediately before irradiation did not recover from the anaesthetic, so it was not possible to assess the response of the tumours to PDT under these conditions.

Fluorescence in frozen sections of tumours was examined 24h after injection of verapamil (10mg/kg) plus HPD. There was an increase in intensity of fluorescence, suggesting verapamil increased uptake or retention of HPD in the tumours.

In control experiments, verapamil only, verapamil plus light, and verapamil plus HPD, all had no effect on the growth of the tumours.

(c). Influence of Noradrenaline on PDT

Methods

Mice with Lewis lung carcinoma were given HPD (50mg/kg i.p.) concurrently with noradrenaline (Levophed, Winthrop; 0.1 or 0.2mg/kg i.v.). These doses of noradrenaline had been previously shown to reduce blood flow in rat tumours [Mattsson et al., 1978]. The tumours were irradiated either 2 or 24h

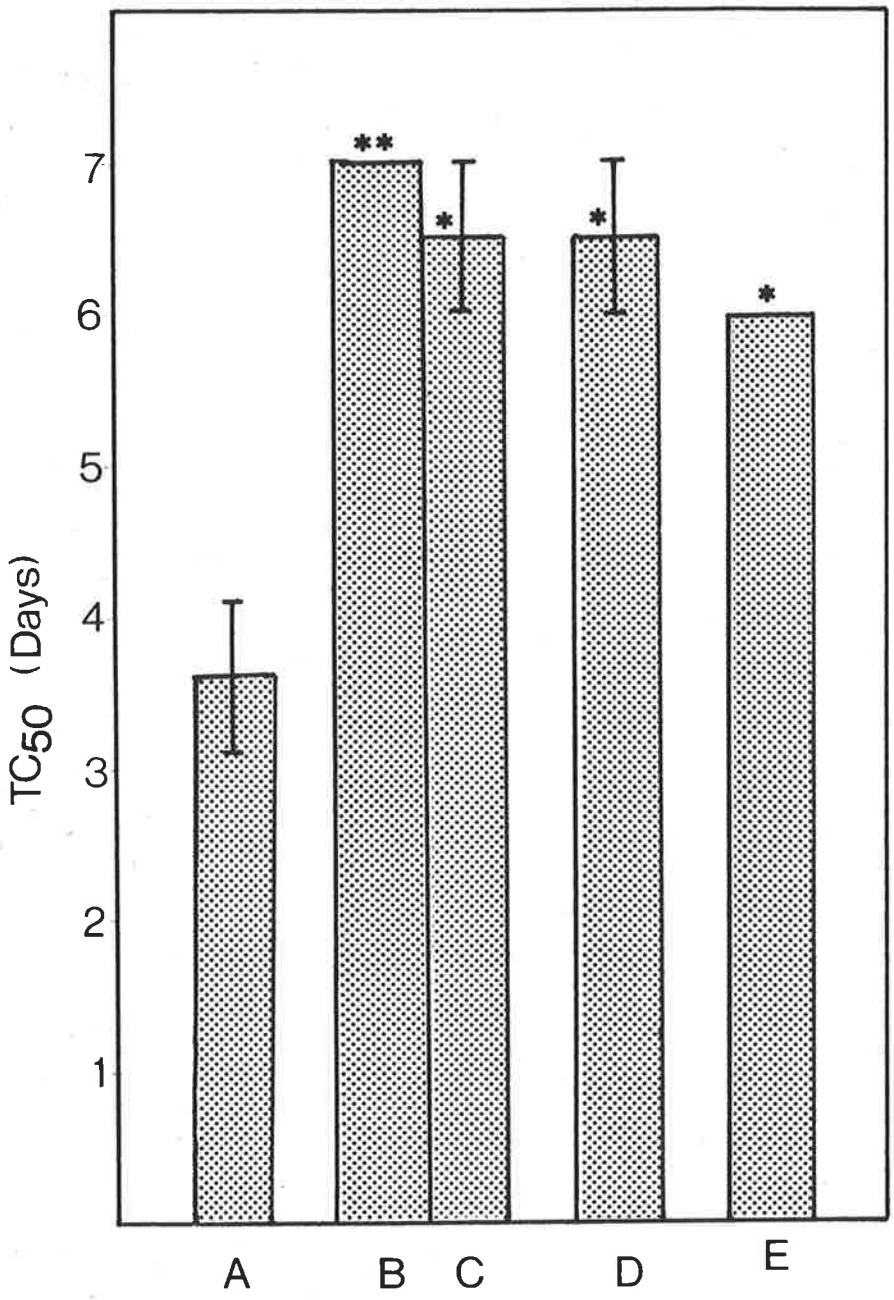


Fig. 9.1

Effect of verapamil on the efficacy of PDT

Mice with Lewis lung carcinoma were given HPD and verapamil and tumours irradiated 24h later. Alternatively, verapamil was administered 24 and 48h after irradiation.

A: HPD (30mg/kg i.p.)

B: HPD plus verapamil (2mg/kg i.p.)

C: HPD plus verapamil (2mg/kg i.v.)

D: HPD plus verapamil (10mg/kg)

E: Verapamil (2mg/kg) administered 24 and 48h after PDT

* $p<0.02$ ** $p<0.01$

after injections. In alternative experiments, mice were given HPD (50mg/kg i.p.) and 24h later, noradrenaline (0.2mg/kg i.v.) was administered approximately 5 min before irradiation.

Noradrenaline (200ul of 0.02mg/ml, 0.2mg/kg total body weight) was injected locally into tumours concurrently with HPD (50mg/kg i.p.). Two hours later, the mice were anaesthetised and the tumours irradiated.

Results

Noradrenaline inhibited the response of the tumours to PDT when administered concurrently with HPD and the tumours irradiated 2h later (Fig. 9.2). However, TC_{50} was not significantly altered by administering noradrenaline concurrently with HPD and irradiating the tumours 24h later (Fig. 9.2). Noradrenaline administered 5min before irradiation also had no effect on the TC_{50}. Local administration of noradrenaline into the tumours concurrently with HPD (i.p.) followed 2h later by irradiation, greatly inhibited the response to PDT. Some of the tumours were still palpable 24h after irradiation (TC_{50} of less than 1 day).

Noradrenaline (0.2mg/kg) alone, noradrenaline plus HPD (50mg/kg) without light, or noradrenaline followed by irradiation of the tumours 5min or 24h later, all had no visible effect on the tumours. Local administration of noradrenaline also had no effect on tumour growth. The mice were lethargic with ruffled fur immediately after i.v. administration of 0.2mg/kg noradrenaline, but recovered within 30min. Higher doses of noradrenaline were not tested.

Noradrenaline inhibited uptake of HPD in tumours when administered concurrently with HPD 2h before tumours were removed. Fluorescence was fainter after 0.2mg/kg noradrenaline

Fig. 9.2

Effect of noradrenaline on the efficacy of PDT

Mice with Lewis lung carcinoma were given HPD i.p. plus noradrenaline i.v. 2h before irradiation. Alternatively, tumours were irradiated 24h after HPD and noradrenaline was administered either with HPD or 5 min before irradiation.

A: HPD (50mg/kg) 2h before irradiation

B: HPD plus noradrenaline (0.1mg/kg) 2h before irradiation

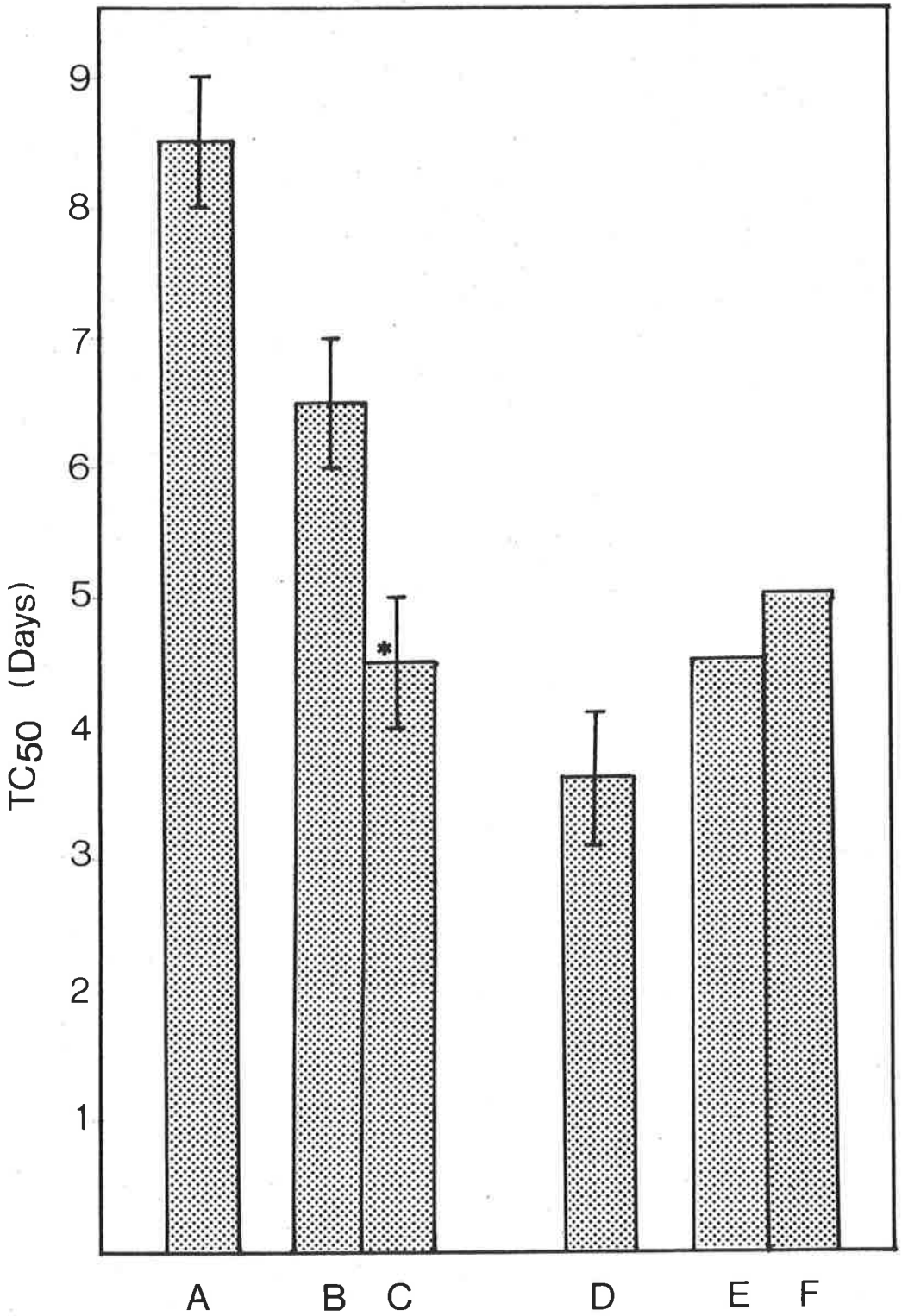
C: HPD plus noradrenaline (0.2mg/kg) 2h before irradiation

D: HPD (30mg/kg) 24h before irradiation

E: HPD 24h before irradiation plus noradrenaline (0.2mg/kg) 5min before irradiation

F: HPD plus noradrenaline (0.2mg/kg) 24h before irradiation.

* $p<0.05$


TC50 (Days)
9
8
7
6
5
4
3
2
1
*
A
B
C
D
E
F

than 0.1mg/kg, which correlated with a greater inhibition of efficacy of PDT. Intensity of fluorescence was not altered by concurrent administration of HPD plus noradrenaline 24h before the tumours were removed. This correlated with a lack of modification of the efficacy of PDT.

(d). Effect of propranolol on PDT

Methods

Mice with Lewis lung carcinoma were given HPD (30mg/kg i.p.) and propranolol (Inderal, ICI Australia; 0.2-1.0mg/kg i.v.) and the tumours irradiated 24h later. Doses of propranolol were chosen to approximate those used clinically. In some experiments, mice were given HPD (30mg/kg) followed 24h later by propranolol (1.0mg/kg i.v.) approximately 5 min before irradiation.

Results

The effect of propranolol on the efficacy of PDT is shown in Fig. 9.3. The results are equivocal, with a statistically significant increase in TC_{50} with 0.5mg/kg propranolol but not with 0.2 or 1.0mg/kg. Propranolol (1.0mg/kg) administered 5 min before irradiation did not alter TC_{50} which remained at 3 days (Fig. 9.3). Higher doses of propranolol were not used as the mice became lethargic immediately after injection of 1.0mg/kg. Propranolol only (1.0mg/kg), propranolol plus HPD and propranolol plus light all had no effect on the growth of tumours.

Intensity of fluorescence in the tumours after injection of HPD was not altered by propranolol, indicating that propranolol had no effect on uptake of HPD into the tumours. This corresponded with a probable lack of effect of propranolol

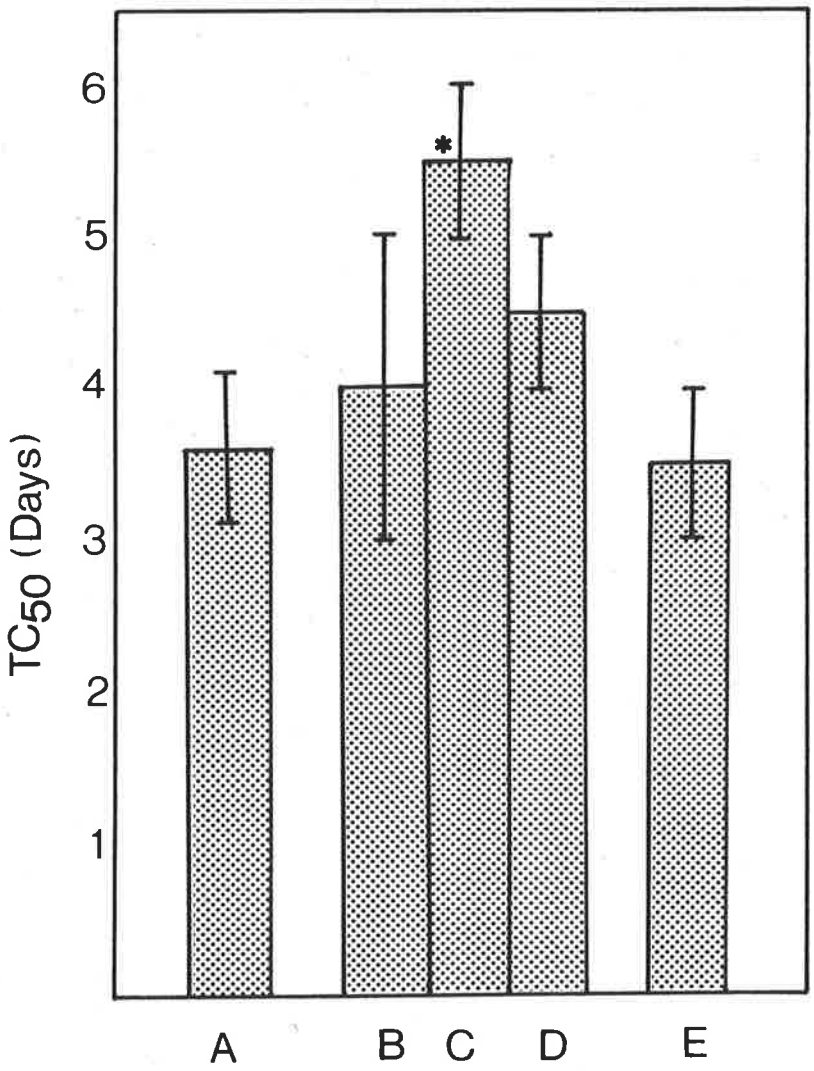


Fig. 9.3

Effect of propranolol on the efficacy of PDT

Mice with Lewis lung carcinoma were given HPD plus propranolol i.v. 24h before irradiation.

A: HPD (30mg/kg)

B: HPD plus propranolol (0.2mg/kg)

C: HPD plus propranolol (0.5mg/kg)

D: HPD plus propranolol (1.0mg/kg)

E: HPD 24h before irradiation plus propranolol (1.0mg/kg) 5min before irradiation.

* $p<0.02$

on the efficacy of PDT.

(e). Influence of hydralazine on PDT

Methods

Mice with Lewis lung carcinoma were given HPD (30mg/kg i.p.) plus hydralazine (Apresoline, Ciba-Geigy, Aust. Ltd.; 5 or 10mg/kg i.v.) and the tumours irradiated 24 h later. Doses of hydralazine were chosen to approximate those in clinical use. In some experiments, mice were given HPD (30mg/kg) followed 24h later by hydralazine (10mg/kg) approximately 5min before irradiation.

Results

Hydralazine, administered either concurrently with HPD or immediately before irradiation, had no effect on the response of the tumours to PDT (Fig. 9.4). Intensity of fluorescence in the tumours 24h after injection of HPD was not altered by hydralazine. This corresponded with a lack of effect of hydralazine on the efficacy of PDT. Hydralazine alone, hydralazine plus HPD without light or hydralazine plus light all had no effect on the growth of the tumours. Immediately after administration of hydralazine (10mg/kg), the mice were very lethargic, so higher doses of hydralazine were not tested.

(f). Blocking the effects of noradrenaline

Noradrenaline may inhibit uptake of HPD and hence efficacy of PDT by its vasoconstricting action. An appropriate vasodilator may block these effects. This was tested by administering intravenously a mixture of noradrenaline and propranolol concurrently with HPD (i.p.) and irradiating the tumours 2h later.

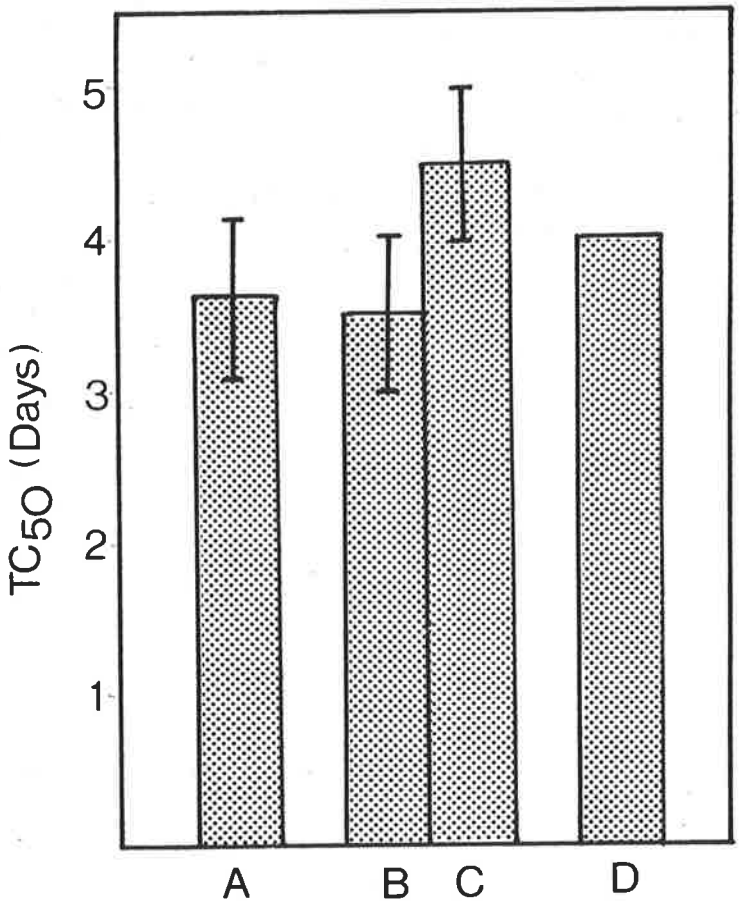


Fig. 9.4

Effect of hydralazine on the efficacy of PDT

Mice with Lewis lung carcinoma were given HPD plus hydralazine i.v. 24h before irradiation.

A: HPD (30mg/kg)

B: HPD plus hydralazine (5mg/kg)

C: HPD plus hydralazine (10mg/kg)

D: HPD 24h before irradiation plus hydralazine (10mg/kg) 5min before irradiation

Method

Mice with Lewis lung carcinoma were given a mixture of noradrenaline (0.2mg/kg) and propranolol (0.5mg/kg) intravenously followed immediately by HPD (50mg/kg i.p.) and the tumours irradiated 2h later. TC_{50} was determined as previously described.

Results

Concurrent administration of noradrenaline and propranolol with HPD did not significantly alter the inhibition of PDT by noradrenaline. TC_{50} was 5.5±0.5 days, compared to 4.5±0.5 days after noradrenaline. PDT without any vasoactive drugs gave a TC_{50} of 8.5±0.5 days.

Concurrent administration of noradrenaline and propranolol greatly inhibited uptake of HPD. Fluorescence intensity was similar to that observed after noradrenaline plus HPD. This corresponded to the degree of inhibition of the efficacy of PDT.

(g). Influence of verapamil on the photoactivity of HPD in vitro

The mechanisms by which verapamil potentiates PDT may result from its vasodilating activity or its calcium channel blocking activity, both of which could increase the uptake or retention of HPD in tumours. Other unknown properties of verapamil may also be involved. To attempt to differentiate between these effects, the influence of verapamil on the photodynamic destruction of tumour cells *in vitro* was examined. Any effects of verapamil *in vitro* will not be due to vasodilation and will be more likely due to the calcium channel blocking activity.

Method

The influence of verapamil on the photocytotoxic activity of HPD *in vitro* was examined using the ^{51}Cr release assay described in Chapter 2. Lewis lung carcinoma cells were grown and harvested *in vitro* as described in Chapter 3. ^{51}Cr-labelled Lewis lung carcinoma cells ($10X10^6$/ml in RPMI 1640) were incubated for 1h at 37^{o} with HPD (25ug/ml) and verapamil (0-100ug/ml). The cells were washed once, resuspended in PBS, irradiated for 0-20min and percentage ^{51}Cr release determined immediately after irradiation as previously described. Background percentage ^{51}Cr release, from cells incubated in HPD or HPD plus verapamil but not irradiated, was subtracted from all experimental values. Uptake of HPD into Lewis lung carcinoma cells was assessed by fluorescence as described in Chapter 2 after incubating cells for 1h in HPD plus graded doses of verapamil.

Results

The lack of effect of verapamil on ^{51}Cr release from HPD-sensitized Lewis lung carcinoma cells is illustrated in Fig. 9.5. The same profiles of ^{51}cr release were observed when cells were incubated with HPD in the presence and absence of graded concentrations of verapamil. However, there was an increase in the intensity of HPD fluorescence after incubating the cells in the presence of verapamil, suggesting increased uptake of HPD without a corresponding increase in photodynamic damage to the cells. Verapamil alone (10-50ug/ml) or verapamil plus light did not cause ^{51}Cr release above background. Higher concentrations of verapamil could not be tested. There was elevated ^{51}Cr release from cells incubated in verapamil only (100ug/ml), indicating that concentrations toxic to the cells had been

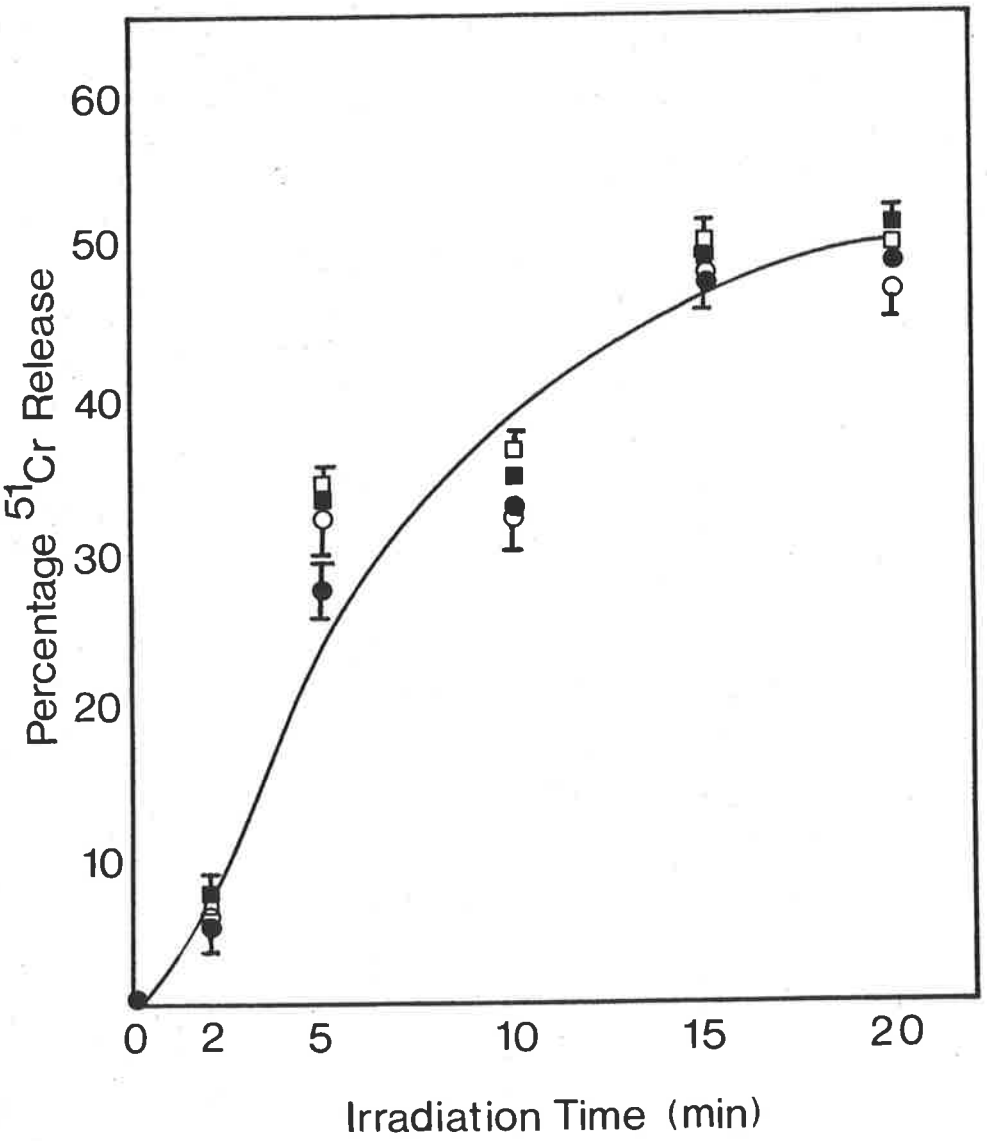


Fig. 9.5

Influence of verapamil on ^{51}Cr release from HPD-sensitized Lewis lung carcinoma cells

^{51}Cr-labelled Lewis lung carcinoma cells were incubated for 1h with HPD (25ug/ml) and verapamil (0-100ug/ml) and irradiated.

● HPD (25ug/ml)

○ HPD plus verapamil (10ug/ml)

■ HPD plus verapamil (25ug/ml)

□ HPD plus verapamil (50ug/ml)

reached.

(h). Discussion

The influence of vasoactive drugs on the efficacy of PDT has been examined using the transplantable tumour model in mice. Verapamil potentiated the uptake of HPD into tumours, resulting in enhanced efficacy of PDT, and, when administered after PDT, also inhibited recurrence of tumours. Noradrenaline inhibited both uptake of HPD and efficacy of PDT, while propranolol and hydralazine had very little effect.

Modification of the state of the tumour blood vessels could alter the efficacy of PDT by a number of mechanisms. These include dilatation or vasoconstriction to modify the rate of blood flow, thereby influencing the amount of HPD that is delivered to the tumours. Alterations in the permeability of the vessel walls may also affect the uptake of HPD. As discussed in Chapter 8, alterations in capillary permeability by glucocorticoids may be responsible for inhibition in HPD uptake. The susceptibility of the vasculature to photodynamic damage could also be altered by vasoactive drugs, leading to altered responses of the tumours to PDT.

The state of dilatation of the tumour blood vessels may be important in regulating uptake of HPD. There is some controversy as to whether tumour blood vessels are able to respond to vasoactive drugs. The blood vessels in the border between rat tumours and muscle showed adrenergic innervation, but no vessels with adrenergic innervation were observed in the tumour tissue [Mattsson et al., 1977]. The morphology of the tumour vasculature varies with the age and histological type of the tumour [Mattsson and Petersen, 1981]. The vascular bed of

young and small tumours consists of a fine capillary bed of normal vessels with intact innervation. As the tumour enlarges, the blood vessels in the tumour become stretched and tortuous, lose adrenergic innervation and show a relative lack of smooth muscle. It is difficult to predict the reactivity of tumour blood vessels to vasoactive drugs.

The influence of vasoactive drugs on tumour blood flow has been examined in a number of systems. Blood flow in rat tumours was reduced by local administration of noradrenaline [Mattsson et al., 1980] and vasoconstriction was observed directly by microangiography [Mattsson et al., 1981]. Tumour blood flow was also reduced by i.v. administration of noradrenaline [Mattsson et al., 1978]. This reduction in blood flow was blocked by phenoxybenzamine (an alpha-receptor blocking agent). In the experiments described above, noradrenaline reduced the uptake of HPD into tumours. This reduction could be due to vasoconstriction of the tumour blood vessels. The inhibitory effect of noradrenaline was not blocked by propranolol, a beta blocking agent. This would be expected, since noradrenaline acts mainly on alpha receptors with little beta receptor activity [Weiner, 1985].

Rather than noradrenaline acting directly on the tumour blood vessels, uptake of HPD into tumours may be blocked indirectly by noradrenaline acting on the blood vessels of the peritoneum to reduce systemic absorption of HPD. Local administration of noradrenaline into the tumours concurrently with HPD (i.p.) was more effective in inhibiting tumour destruction than i.v. administration of noradrenaline. This may reflect a greater local concentration of noradrenaline in the

tumour. Vasoconstriction of the tumour blood vessels or surrounding vasculature must be involved in determining efficacy of uptake of HPD. However, vasoconstriction of other normal blood vessels in the body could also affect delivery and uptake of HPD into the tumour.

The ability of tumour blood vessels to undergo vasodilation is controversial. The tumour vasculature may be maximally dilated because of a lack of contractile elements in the vessel walls and therefore will be insensitive to pharmacological agents. This was suggested by a lack of reactivity of tumour vasculature to isoprenaline, papaverine and dihydralazine [Petersen and Mattsson, 1984]. The response of tumour vasculature to vasoactive drugs, including propranolol, decreased as the tumours enlarged [Wickersham et al., 1977]. The lack of effect of propranolol and hydralazine on the uptake of HPD and hence on the efficacy of PDT could be due to the inability of the tumour blood vessels to respond to drugs if they are already in a state of maximum vasodilation. Hydralazine acts by directly relaxing vascular smooth muscle [Rudd and Blaschke, 1985] so tumour blood vessels with a lack of smooth muscle would not be expected to be responsive to hydralazine.

Administration of noradrenaline, propranolol or hydralazine immediately before irradiation did not alter the TC_{50}. The influence on PDT of verapamil administered immediately before irradiation could not be tested, as the combination of verapamil and anaesthetic was lethal to the mice. Therefore, the state of the tumour vasculature at the time of irradiation did not appear to influence the outcome of PDT.

It will be necessary to develop methods to assess directly changes in the tumour blood vessels to confirm the role of vasoactive drugs in modifying responses to PDT. The present studies do not demonstrate directly whether the tumour blood vessels are altered by the drugs. Tumour blood flow measurements by radiolabelled microspheres [Selman et al., 1984], quantitative microscopy or microangiography [Petersen and Mattsson, 1984] would provide information on the state of the tumour vasculature. Without these measurements, the mechanisms by which vasoactive drugs influence the efficacy of PDT cannot be proved.

Verapamil enhanced the efficacy of PDT by increasing uptake or retention of HPD in the tumours. It also delayed recurrence of tumours when administered after PDT. Both these interactions may be very useful clinically to improve the efficacy of PDT. Possible mechanisms for these effects include the calcium channel-blocking activity or the vasodilating activity of verapamil.

Verapamil may act as a peripheral vasodilator in humans [McMahon and Sheaffer, 1982]. However, its ability to alter tumour blood flow is controversial. Tumour blood flow was increased by verapamil but without altering blood flow in normal rat tissue, suggesting verapamil could be used to enhance delivery of chemotherapeutic drugs to tumours [Kaelin et al., 1982]. In contrast, Robinson et al. [1985] did not detect enhancement by verapamil of tumour blood flow in a mouse fibrosarcoma. It is questionable whether the potentiation of PDT by verapamil is due to its vasodilating activity. The other vasodilators tested, propranolol and hydralazine, had no effect on uptake of HPD and hence on the efficacy of PDT, suggesting

that vasodilation does not play a role in increasing the efficacy of PDT. Vasodilation of the tumour vasculature is also unlikely to be involved in the inhibition by verapamil of tumour recurrence after PDT.

The calcium channel blocking activity of verapamil appears more likely than vasodilation to be the cause of the increase in uptake or retention of HPD in tumours. There have been a number of reports of verapamil either enhancing the effect of cytotoxic drugs or converting cytotoxic drug resistance to sensitivity. Verapamil enhanced the cytotoxic effect of daunorubicin on Ehrlich ascites carcinoma cells [Slater et al., 1982]. Tumour cell lines (including Lewis lung carcinoma) resistant to vincristine and Adriamycin in vitro were made sensitive by verapamil [Tsuruo et al., 1982; 1983a; 1983b; 1983c]. The intracellular concentrations of cytotoxic drugs were elevated, suggesting that verapamil inhibited outward transport of drugs from the cells. The conversion to drug sensitivity by verapamil was also observed in mouse tumours treated with vincristine [Tsuruo et al., 1983a; 1983c; 1985]. Verapamil (10mg/kg) prolonged growth delay of two mouse tumours by melphalan [Robinson et al., 1985]. Potentiation of cytotoxic drugs by verapamil was also shown in human tumours. The destruction by Adriamycin of human bladder cancer cells [Simpson et al., 1984] and ovarian carcinoma cells [Rogan et al., 1984] was potentiated by verapamil. A clinical trial to examine the effect of verapamil on the treatment of Adriamycin-resistant ovarian carcinoma has been commenced [Rogan et al., 1984].

Studies in vitro indicated that verapamil did not

influence the degree of photodynamic damage to Lewis lung carcinoma cells. Increased HPD fluorescence was noted in cells incubated in HPD plus verapamil but this was not reflected in increased ^{51}Cr release. This discrepancy could not be explained but may reflect the inadequate methods for assessing HPD uptake. There was therefore no evidence that verapamil acts directly on tumour cells to increase photodynamic damage. The site of action of verapamil may be in the cells of the vasculature. Rather than increasing HPD uptake by vasodilation, verapamil may increase permeability of the blood vessels to HPD by calcium channel blocking and also inhibit efflux of HPD from the cells of the vascular stroma. In this manner, the vascular system would be more susceptible to photodynamic damage, leading to increased response to PDT.

When administered after PDT, verapamil also delayed recurrence of tumours. Elevated intracellular calcium levels may inhibit repair of photodynamic damage and regrowth of both the tumour cells and the supporting vascular system. It is unlikely that vasodilation would play a role in inhibiting repair of the tumour or the tumour vasculature. Alternatively, a yet unidentified property of verapamil may be involved in these interactions.

In conclusion, the major effect of the vasoactive drugs appears to be the modification of uptake of HPD, resulting in altered efficacy of treatment. Further studies are needed to define precisely the effect of the vasoactive drugs on the state of the tumour vasculature and their influences in modifying the outcome of PDT.

CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

The photodynamic and tumour-localizing properties of HPD are used for the treatment of malignant tumours in man. As discussed in Chapter 1, the therapeutic role of photodynamic therapy (PDT) has not yet been adequately defined. Tumours which respond to PDT with a therapeutically useful result have been identified. Methods have been developed to deliver light to the tumours. Dosimetry, both for HPD and light, is gradually being refined.

PDT is a local treatment and necrosis of the tumour is limited to the area that receives an effective light flux. Effective penetration of red light through tissues is variable but is probably less than 10mm with presently available light sources [Dougherty et al., 1985]. Large tumour masses cannot be effectively treated by a single irradiation. Even if repeated administration of light caused necrosis of a large tumour, ulceration and poor healing could mean that the treatment would not be of practical help to the patient. Therefore the tumours most appropriate for PDT are small localized tumours.

Another major problem that limits the efficacy of PDT is the relatively poor therapeutic ratio. This is a reflection of the relatively low selectivity of uptake of HPD into tumours compared to normal tissues. Since normal tissues are photosensitized, adjacent structures may also be damaged during irradiation. Haemorrhage could occur if there was necrosis of underlying blood vessels during PDT. The high levels of porphyrin in the skin cause severe cutaneous photosensitivity. This limits the application of PDT to patients where the

benefits of treatment outweigh the problems associated with photosensitivity. The efficacy of PDT would be greatly improved if the therapeutic ratio between tumours and normal tissue could be increased. Higher concentrations of HPD in the tumours would result in more effective treatment. Alternatively, a lower total dose of HPD could be administered to the patient, resulting in the same concentration of HPD in tumours but much less in normal tissues.

Efficacy of treatment will be improved both by increasing the degree of initial tumour destruction and by reducing the rate of recurrence of tumours after PDT. PDT should be considered in the context of other anti-cancer treatments. A single treatment modality is not always used. Patients are often treated with a combination of surgery, radiotherapy or chemotherapy. A combination of chemotherapeutic drugs is also commonly used. The most effective use of PDT may be as one of a combination of treatment modalities. Individually, each treatment may have inadequate effects but the combination may provide good initial tumour clearance and a reduction in the rate of recurrence of tumours. This may also allow lower doses of cytotoxic drugs or radiotherapy to be used with a reduction in morbidity.

In this thesis, a number of approaches to improving the efficacy of PDT have been examined.

(1). Improvement of the specificity of uptake of HPD into tumours compared to normal tissue. This improvement could be achieved by finding porphyrins with superior tumour-localizing abilities. Alternatively, HPD could be targeted into a tumour by using a tumour-specific antibody.

(2). Use of a combination of the cytotoxic effects of PDT with those of other agents to obtain enhanced tumour destruction.

(3). Improvement of the therapeutic effect of PDT. This could be achieved in several ways:

(i). Enhancement of the uptake of HPD into tumours.

(ii). Reduction of the efflux of HPD from tumours.

(iii). Improvement in the sensitivity of the tumour to photodynamic damage.

(iv). Reduction in the rate of regrowth of tumours.

The chemical composition of HPD was examined to identify the phototoxic and tumour-localizing porphyrins. The removal of inactive porphyrins from HPD may allow a lower total dose of porphyrin to be administered, while still resulting in the same concentration of porphyrin in the tumour. This may possibly reduce cutaneous photosensitivity. The tumour-localizing and photodynamic properties of HPD in a transplantable mouse tumour were confined to a highly-aggregated hydrophobic component. This fraction resulted in the same degree of tumour destruction and skin photosensitization as HPD itself. Specificity of uptake of HPD into tumours and the efficacy of PDT was not improved by this approach.

The tumoricidal properties of another porphyrin, porphyrin C, were examined. Porphyrin C caused tumour destruction to the same extent as HPD but had the advantage of being cleared very quickly from the mouse. Therefore, while the initial tumoricidal effect and cutaneous photosensitization were the same as with HPD, the duration of photosensitivity would be greatly reduced. New porphyrins with different rates of uptake and clearance may be preferable to HPD.

The antibody-targeted delivery of porphyrins to tumours was examined as a method of improving the specificity of uptake of HPD. It has been claimed in previous reports that antibody-directed delivery of HP to tumours results in improved tumour destruction after irradiation. An in vitro model was developed to assess any potential improvement in the specificity of uptake of porphyrin. The HP-antibody conjugate was phototoxic in vitro, but the specificity of binding of the antibody to surface immunoglobulin on B lymphocytes was destroyed. The reasons for this are not clear. HP may cause alterations in the conformation of the antibody molecule, destroying antigen-binding sites. Binding of porphyrin to its natural binding sites on the cells persisted, rather than binding of the conjugate to cells being directed by the antibody specificity of the conjugate. This appears to be a large problem in using antibody-guided delivery of porphyrins and discouraged further attempts to utilize this approach.

Pharmacological manipulation may be the most useful method of improving the efficacy of PDT. The combination of the cytotoxic effects of PDT and chemotherapeutic drugs caused improved tumour destruction. Adriamycin and methotrexate were both synergistic with PDT. Since many patients are being treated concurrently with other drugs, it is also important to document interactions between PDT and other agents. In this way, the role of PDT as one of a range of anti-cancer treatments may be defined.

Improvements in uptake of HPD with a corresponding increase in the efficacy of PDT has been achieved in several ways. Uptake of HPD in tumours (assessed by fluorescence) was

increased when other drugs were administered concurrently with HPD. Adriamycin, methotrexate, low doses of methylprednisolone acetate and verapamil were all effective in this way. Although the uptake of HPD into tumours was increased, cutaneous photosensitivity persisted. The increase in fluorescence could also have resulted from inhibition by the drugs of efflux of HPD from the tumours. The improvement in uptake of HPD and in the duration of tumour control offers the possibility of adequate therapy with lower doses of HPD and a reduction in cutaneous photosensitivity.

Some drugs must be avoided since they reduce the uptake of HPD into tumours and inhibit the response of tumours to PDT. The concurrent administration of high doses of methylprednisolone acetate or noradrenaline with HPD greatly inhibited uptake of HPD into tumours, thereby inhibiting PDT.

Efficacy of PDT will be improved if there is a reduction in the rate of recurrence of tumours after PDT. Administration of glucocorticoids after PDT greatly increased the duration of tumour control in mice. A number of possible mechanisms were considered. Glucocorticoids may prevent repair of the tumour vasculature or alternatively slow the rate of regrowth of tumour cells. This new approach requires further extensive investigation as it could be very useful in the clinical situation. Glucocorticoids could be administered for a few days after PDT to minimise recurrence of tumours.

Pharmacological manipulations may also be useful in examining mechanisms of tumour destruction by PDT. The vasoactive drugs may be used to manipulate the state of the tumour vasculature and determine its role in localization of HPD and in the destruction of tumours. The vasoconstricting

drug, noradrenaline, inhibited uptake of HPD and reduced the efficacy of PDT but the vasodilators, propranolol or hydralazine had no effect. Alteration of the state of the vasculature could increase the sensitivity of the tumour vasculature to photodynamic damage and hence increase the efficacy of tumour destruction. Inhibition of regrowth of the vasculature by pharmacological agents may also inhibit repair and regrowth of the tumour cells. Future studies, which are outside the scope of this thesis, will involve the development of models for the measurement of tumour blood flow. These measurements will give a more direct measure of the effects of vasoactive drugs on the tumour vasculature and on photodynamic destruction of tumours.

In conclusion, these studies show clearly that the efficacy of PDT may be influenced greatly by the concurrent use of other pharmacological agents. This may occur by a number of mechanisms. HPD uptake into tumours may be increased, efflux of HPD from tumours may be inhibited, recovery from sublethal damage and replication of tumour cells may be inhibited, regrowth of the tumour vasculature could be inhibited, and the susceptibility of tumour cells and vasculature to photodynamic damage may be increased. This opens up a previously unexplored area of investigation with considerable therapeutic potential. The role of PDT in cancer treatment may not be as an isolated modality except in small superficial tumours but as one of a combination of available treatments for cancer.

APPENDIX A

ABBREVIATIONS

AET: aminoethylisothiouronium bromide

BSA: bovine serum albumin

CHO: Chinese hamster ovary cells

^{51}Cr: chromium-51

D_2O: deuterium oxide

ESR: electron spin resonance

FCS: foetal calf serum

HEPES: N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid

HP: haematoporphyrin dihydrochloride

HPD: haematoporphyrin derivative

HPLC: high performance liquid chromatography

HVD: hydroxyethyl vinyl deuteroporphyrin

H_2O_2: hydrogen peroxide

i.p.: intraperitoneal

i.v.: intravenous

J: joules

LDL: low density lipoprotein

mW: milliwatts

NADPH: dihydronicotinamide adenine dinucleotide phosphate

NBT: nitroblue tetrazolium

NMR: nuclear magnetic resonance

$^{\bullet}OH$: hydroxyl radical

$^1O_2$: singlet oxygen

O_2^-: superoxide anion

O.D.: optical density

PAGE: polyacrylamide gel electrophoresis

PBS: Dulbecco's phosphate buffered saline

PDT: Photodynamic therapy

PP: protoporphyrin

S.D.: standard deviation

SDS: sodium dodecyl sulphate

SOD: superoxide dismutase

TC_{50}: time in days for 50% recurrence of tumours after PDT

TLC: thin layer chromatography

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PHOTOCYTOTOXICITY IN VIVO OF HAEMATOPORPHYRIN DERIVATIVE COMPONENTS

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SUMMARY

Phototoxicity of haematoporphyrin derivative (HPD) and some of its component porphyrins was assayed using Lewis Lung Carcinoma transplanted in C57BL mice. Skin photosensitivity was assessed by measuring percentage increase in footpad thickness after exposure to light.

HPD and its aggregate fraction photosensitised the skin, was active in tumour destruction and caused fluorescence in the tumour while the non-aggregate fraction and pure haematoporphyrin were inactive, both in treatment of tumours and in sensitising the skin. The commercial product, Photofrin II was also photoactive and protoporphyrin was slightly active.

It is concluded that the therapeutic activity of HPD is associated entirely with the aggregate fraction and compounds effective in tumour phototherapy also sensitise the skin.

INTRODUCTION

Photochemotherapy with HPD has been used in the treatment of malignant tumours in man [1,2,3,7,8]. Intravenously injected HPD is retained selectively in tumours and is activated by red light (approx. 630 nm). Tumour destruction has been attributed to release of singlet oxygen [4,5,6]. The only major side effect so far reported is skin photosensitivity.

HPD is a complex mixture of porphyrins which can be separated into several fractions by polyacrylamide gel chromatography. The activity as measured in vitro [9] and in vivo [10] is concentrated in a fast-running fraction. The aggregated porphyrins show greatest phototoxicity in a tissue culture assay and porphyrins with vinyl side chains also show greater phototoxicity [11]. We now report studies of the phototoxicity and skin sensitizing capac-

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ity of porphyrins in a transplantable mouse tumour model, and skin photosensitivity using a foot pad assay.

MATERIALS AND METHODS

Mouse tumour assay

Lewis lung carcinoma cells (Dr. L. Dent, Flinders Medical Centre), were transplanted into the back of C57BL mice by subcutaneous injection of approximately 10^6 cells per mouse. After 7—10 days, when the tumours were 5—7 mm in diameter, mice in groups of 10 were given porphyrins (25—60 mg/kg, i.p. or i.v.). Twenty four hours later mice were anaesthetised with Sagatal (May and Baker Aust. Pty. Ltd.), the fur over the tumour shaved and a 1-cm diameter area over the tumour was irradiated with red light as specified. Mice were palpated daily for recurrence of tumour. The end point was the number of days for 5 out of 10 mice to regrow palpable tumour. The assay was modified from Dougherty [10].

Preparation of porphyrin fractions

HPD [2], haematoporphyrin, protoporphyrin, HPD aggregate and HPD non-aggregate were prepared and characterised as previously described [11] and administered in isotonic saline. Photofrin II was supplied by Dr. T.J. Dougherty, Roswell Park Memorial Institute. The concentration was determined by measuring OD_{397} of a solution of porphyrin diluted in 50% ethanol/0.1 N NaOH.

Skin photosensitivity

Mice in groups of 10 were given porphyrins i.p. and the left footpad was irradiated 24 h later with a light dose of 112 J/cm^2. Twenty four hours later the thickness of both feet was measured with a micrometer and percentage increase in footpad thickness was calculated using the right foot as an untreated control.

Light sources

A gold metal vapour laser [12] (Quentron Optics Pty, Ltd) with wavelength 627.8 nm was used to generate average light intensities of 400 mW, coupled to a 400 μm quartz fibre and positioned to create a 1-cm diameter spot.

An incandescent filament lamp [13] fitted with a perspex lens 10 mm in diameter and 50 mm length delivered 2.5 W uniformly over 1 cm at 620—720 nm wavelength. The effective light flux at 630 nm was 890 mW, determined from the relative light flux absorbed by HPD at 630 nm (Wilksch, 1982, unpublished data). The skin of the mouse was sprayed with water and irradiated in 50-s exposures with 10-s pauses to prevent thermal effects.

Fluorescence of tumours

Tumours were removed 24 h after injection of porphyrin and snap frozen.

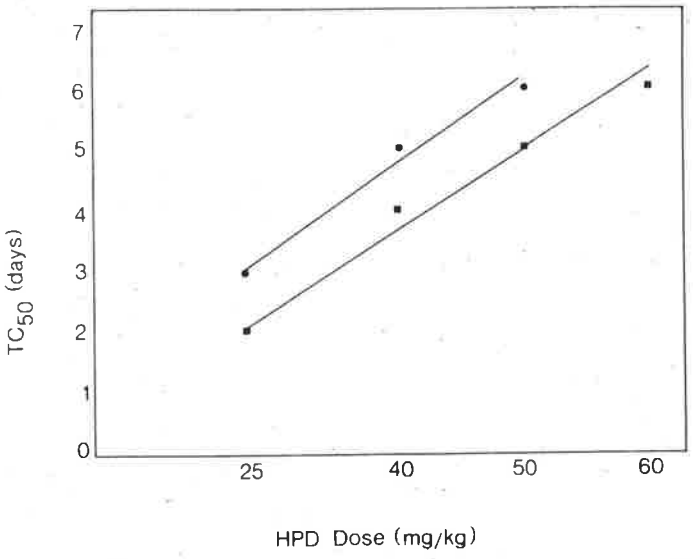

Fig. 1. Relationship between TC_{50} and HPD dose. Mice were given 25—60 mg/kg HPD either i.v. (•) or i.p. (▪). Light dose was 225 J/cm². Each point represents the mean of 2 experiments.

Sections (6 μm) were cut at −14°C on an IEC microtome and examined under a Zeiss fluorescence microscope with excitation wavelength 420—490 nm.

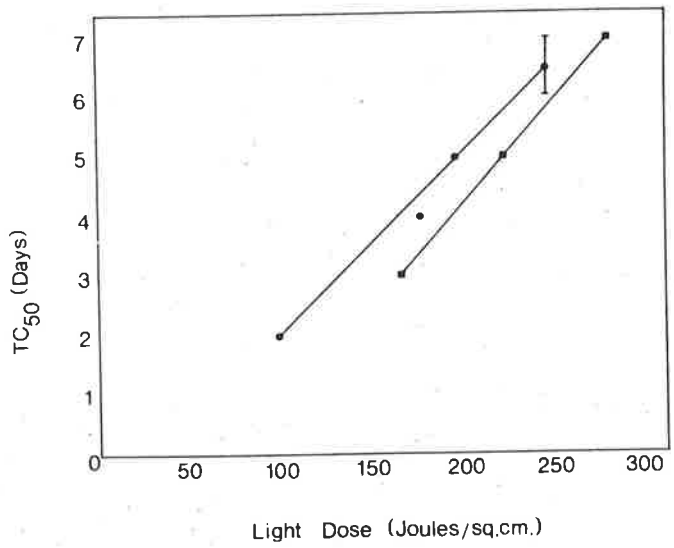

Fig. 2. Relationship between TC_{50} and light dose. Mice were given 50 mg/kg HPD i.p. and tumours irradiated with increasing light doses from either the gold laser (•) or the incandescent filament lamp (▪). Each point represents the mean of 2 experiments.

RESULTS

Relationship between TC_{50} and HPD dose (Fig. 1).

There was a linear relationship between TC_{50} and HPD dose. The TC_{50} for HPD given i.v. was 1 day greater than when given i.p. for all doses tested. Since the difference was not large, i.p. injections were used routinely.

Relationship between TC_{50} and light dose (Fig. 2)

There was a linear relationship between TC_{50} and light dose, with either lamp or gold laser. The gold laser is slightly more efficient than the lamp.

Histology

Tumours 24 h after treatment with HPD and light showed extensive necrosis (Fig. 3a) and isolated viable cells. In contrast, tumours treated with HPD or light alone or untreated showed viable cells with small areas of necrosis (Fig. 3b).

Fluorescence studies

Twenty four hours after i.p. injection of porphyrin, frozen sections of tumours from animals given HPD, HPD-aggregate and Photofrin II showed extensive red fluorescence. Tumours from animals receiving HPD non-aggregate fluoresced weakly (Table 1). Protoporphyrin and haematoporphyrin did not result in any fluorescence in the tumours.

Relative efficiencies of components of HPD in vivo (Table 1)

Haematoporphyrin and HPD non-aggregate caused no tumour phototoxicity under the conditions tested. Increasing the light dose to 300 J/cm^2 also gave no response. HPD aggregate and Photofrin II caused the same phototoxicity as HPD. Protoporphyrin i.p. and i.v. showed very little photoactivity. The photoactivity corresponded with the fluorescence of porphyrin within the tumours.

Skin photosensitivity (Table 1)

HPD, HPD aggregate and Photofrin II all induced approximately the same photosensitivity while HPD non-aggregate, haematoporphyrin and protoporphyrin did not photosensitise the skin.

DISCUSSION

This paper describes the measurement of phototoxicity of HPD and some of its component porphyrins in vivo, using Lewis lung carcinoma transplanted subcutaneously into C57BL mice, measuring the time to 50% recurrence of tumours after treatment (TC_{50}). The TC_{50} was proportional to both the light dose and the porphyrin dose.

Studies with SMT-F mammary tumour in BALB/c mice have shown that

Fig. 3. (a) Histological section of Lewis Lung carcinoma before treatment with HPD and light (400× magnification). (b) Histological section of tumour 24 h after treatment with 50 mg/kg HPD and 200 s irradiation with the filament lamp (400× magnification).

TABLE 1

Porphyrin	TC_{50} (days)	% increase in footpad thickness	Tumour fluorescence
HPD	5	79 ± 21	Positive
HPD Aggregate	5	69 ± 9.9	Positive
HPD Non-aggregate	0	0.8 ± 6.7	Weak positive
Haematoporphyrin	0	0 ± 2.0	Negative
Protoporphyrin	0	−0.2 ± 3.0	Negative
Photofrin II	6	55.4 ± 11.5	Positive

All mice were injected with 50 mg/kg porphyrin i.p. and tumours were irradiated with 225 J/cm² light from the filament lamp, 24 h post injection. For skin photosensitivity, the left footpad was irradiated with 110 J/cm². Results are mean values of at least 2 experiments.

doses of 3.5—10.0 mg/kg HPD were effective [10]. We found 50 mg/kg was necessary to obtain a satisfactory tumour response, in agreement with Ellingsen [14] who successfully treated Lewis lung carcinoma in B_6D_2 mice, using a regime of 50 mg/kg HPD and 160—360 J light at 630 nm.

The aggregated fraction of HPD contained the photocytotoxic activity in vivo. Non-aggregated HPD and haematoporphyrin had no activity. This confirms reports by other authors [10,15]. The activity correlated with the uptake of porphyrin as demonstrated by fluorescence of frozen sections. Similarly, skin photosensitivity was only demonstrated with porphyrins that also caused tumour fluorescence and destruction. This suggests that the critical requirement for in vivo phototoxicity is uptake into the tumours, and HPD non-aggregate, haematoporphyrin and protoporphyrin cannot be taken up into the tumours. Protoporphyrin is photoactive in vitro, [11,16] implying it can be taken up into cells in tissue culture but the mechanism for the selective uptake and retention in the tumour must be lacking in mice.

Photofrin II has been postulated to be the active component of HPD [10]. It exists in aqueous solution in a very highly aggregated form, demonstrated by gel chromatography [10] and spectroscopic data [17]. Dougherty [10] reported a greater tumour phototoxicity using Photofrin II than HPD. We found Photofrin II had similar phototoxic activity to both HPD and HPD aggregate prepared by ultrafiltration. This discrepancy may reflect the different tumour systems used. HPD aggregate and Photofrin II also caused similar skin photosensitivity to HPD. Gomer and Razum [18] also reported that HPD and Photofrin II caused comparable acute skin damage.

Carpenter [19] reported stronger fluorescence and greater tumour selectivity when HPD was injected i.v. compared to i.p. in mice with transplanted mammary adenocarcinoma. Tomio [20] reported equivalent tumour responses in Yoshida ascites hepatomas in rats with intravenous or intraperitoneal

injection of commercial haematoporphyrin. In our studies intravenous HPD had a slightly greater photocytotoxic effect than intraperitoneal HPD, although no marked difference was detected in the fluorescence in the tumours. This probably reflects insensitivity of visual detection of fluorescence.

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POTENTIATION OF PHOTODYNAMIC THERAPY WITH HAEMATOPORPHYRIN DERIVATIVES BY GLUCOCORTICOIDS

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SUMMARY

The effect of glucocorticoids on tumour destruction by photodynamic therapy (PDT) with haematoporphyrin derivative (HPD) and light has been examined in a transplantable mouse tumour model. Administration of glucocorticoid after irradiation enhanced the effect of PDT on both Lewis lung carcinoma and B16 melanoma. Administration of methylprednisolone acetate in depot form concurrently with HPD inhibited the response to PDT while soluble hydrocortisone sodium succinate had no effect. Correctly timed administration of glucocorticoid may have a place in treatment of human tumours by PDT with HPD. Glucocorticoid did not reduce the temporary photosensitivity of the skin induced by HPD.

INTRODUCTION

Photodynamic therapy with HPD relies on selective uptake and retention of HPD in malignant tissue and activation by light of wavelength 620—640 nm [9,10,12]. The precise mechanism of tumour destruction is unknown but singlet oxygen and other oxy-radicals have been implicated [19,26]. Results have been encouraging, particularly in the treatment of lung carcinoma [2,7,15] and bladder carcinoma [3,18].

Since many of the patients undergoing PDT may be treated concurrently with glucocorticoids, their effects on tumour responses and the side effect of skin photosensitivity were studied.

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METHODS

Mouse tumour assay

Photocytotoxic activity of HPD in vivo was assayed using a previously reported method [8]. Two tumours, Lewis lung carcinoma and B16 melanoma, were maintained by passaging through male or female C57BL mice. Mice were 8—12 weeks old, weighed approximately 25 g and were maintained on M and V formula mouse pellets (Milling Industries, Adelaide, S.A.) and water ad libitum. Mice in groups of 10 were given HPD, 30 mg/kg i.p., 7—10 days after subcutaneous injection of 10^6 cells, when tumours were 5—7 mm in diameter. Twenty four hours later the tumours were irradiated with an appropriate dose of red light. Glucocorticoids were administered i.p. either concurrently with the HPD and at the time of treatment or 24 h and 48 h after treatment. Unless otherwise indicated, all mice received 2 doses of glucocorticoid. Tumours were then palpated daily and the time for 5 out of 10 to recur (TC_{50}) was determined. The difference in TC_{50} between groups treated with glucocorticoids or HPD and light only was analysed by an unpaired *t*-test.

Drugs

HPD was prepared as previously described [12]. Hydrocortisone sodium succinate (Efcortelan, Glaxo), methylprednisolone sodium succinate (Solumedrol, Upjohn) and methylprednisolone acetate (Depo-medrol, Upjohn) were prepared as directed by the manufacturer and diluted in sterile saline before i.p. injection at doses stated below. Doses of equivalent potency of hydrocortisone and methylprednisolone were calculated from Ref. 16.

Light sources

An incandescent filament lamp [8] was used to irradiate the tumours with red light (610—680 nm) at the doses shown below.

Skin photosensitivity

Mice with Lewis lung carcinoma were given 30 mg/kg HPD and 3 mg/kg methylprednisolone acetate i.p. Twenty four hours later the left footpad was irradiated with red light for 200 s. Footpad thickness was measured 24 h after treatment with a micrometer and the percentage increase compared to the untreated right foot was calculated.

Fluorescent detection of HPD uptake

Mice with Lewis lung carcinoma were given 30 mg/kg HPD and 0.6—3.0 mg/kg methylprednisolone acetate or 15 mg/kg hydrocortisone sodium succinate. Mice with B16 melanoma were given 30 mg/kg HPD and 3.0 mg/kg methylprednisolone acetate or 15 mg/kg hydrocortisone sodium succinate. Twenty four hours later the mice were killed and fluorescence examined as previously described [8].

RESULTS

Relationship between light dose and TC_{50} for B16 melanoma

There was a linear relationship between TC_{50} and the light dose (Fig. 1), indicating that the TC_{50} is a valid measure of the efficacy of treatment. This relationship was previously seen with the Lewis lung carcinoma [8].

Administration of glucocorticoids before irradiation

The results in Fig. 2 show a linear relationship between dose of methylprednisolone acetate administered before irradiation and TC_{50}. Lewis lung carcinoma is impalpable 24 h after the standard treatment schedule but when methylprednisolone acetate (3.0 mg/kg) was also administered, half the tumours were impalpable, resulting in a TC_{50} of 1 day. At 2.0 mg/kg there was less inhibition and at 1.0 and 0.6 mg/kg the tumour response was augmented. Mice treated with methylprednisolone acetate alone, methylprednisolone acetate + HPD or methylprednisolone acetate + light showed no reduction in tumour size. When methylprednisolone acetate (3.0 mg/kg) was administered at the time of transplant with Lewis lung carcinoma there was no change in the interval before tumours were first palpable, indicating that methylprednisolone acetate alone had no effect on the rate of tumour growth (data not shown).

Hydrocortisone sodium succinate (15 mg/kg) administered before irradiation to mice with Lewis lung carcinoma did not alter the tumour response with TC_{50} of 3 days.

Hydrocortisone sodium succinate administered with HPD and at the time of irradiation did not alter the efficacy of treatment of B16 melanoma, but methylprednisolone acetate at these times caused marked inhibition of

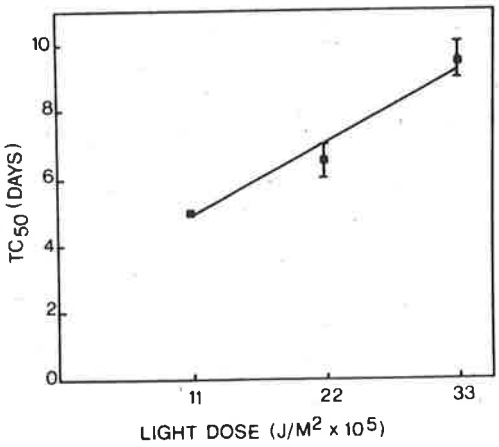

Fig. 1. Relationship between TC_{50} for B16 melanoma and light dose. Mice with B16 melanoma were given HPD, 30 mg/kg i.p. and 24 h later, tumours were irradiated with increasing doses of red light. Each point represents the mean of 2 experiments.

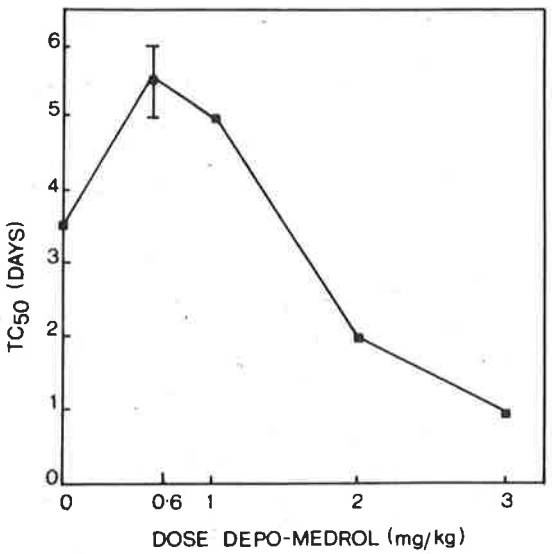

Fig. 2. Relationship between dose of methylprednisolone acetate and TC_{50}. Mice with Lewis lung carcinoma were given HPD, 30 mg/kg plus methylprednisolone acetate i.p. Twenty four hours later, a further dose of methylprednisolone acetate was given and the tumours treated with 200 s light.

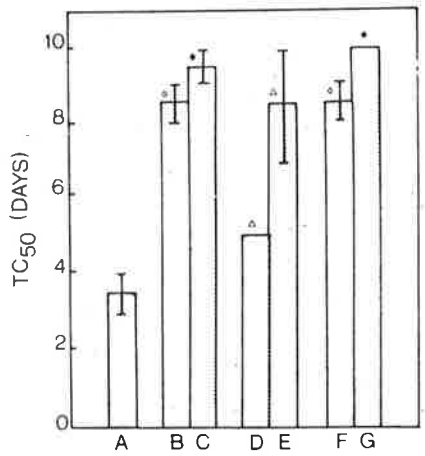

Fig. 3. Administration of glucocorticoids after PDT. Mice with Lewis lung carcinoma were given HPD, 30 mg/kg followed 24 h later by 200 s irradiation to tumours. Glucocorticoids were administered i.p. 24 h and 48 h after treatment. (A) No glucocorticoid; (B) methylprednisolone acetate, 0.6 mg/kg; (C) methylprednisolone acetate, 3.0 mg/kg; (D) methylprednisolone sodium succinate, 0.6 mg/kg; (E) methylprednisolone sodium succinate, 3.0 mg/kg; (F) hydrocortisone sodium succinate, 3.0 mg/kg; (G) hydrocortisone sodium succinate, 15.0 mg/kg. △, $P < 0.05$; ◇, $P < 0.01$; ♦, $P < 0.001$.

tumour response (Fig. 4). Hydrocortisone sodium succinate alone, hydrocortisone sodium succinate + HPD, or hydrocortisone sodium succinate + light treatment had no visible effect on tumour growth. When hydrocortisone sodium succinate was administered at the time of transplantation, the time for appearance of tumour did not change (data not shown).

Administration of glucocorticoids after irradiation

All glucocorticoids had a highly inhibitory effect on the rate of recurrence of Lewis lung carcinoma (Fig. 3). Hydrocortisone sodium succinate was the most effective; methylprednisolone acetate was more effective than methylprednisolone sodium succinate, suggesting that a longer lasting form of glucocorticoid may be more effective.

Recurrence of B16 melanoma was also inhibited by administration of hydrocortisone sodium succinate after irradiation (Fig. 4). The effect was not as marked as that observed with Lewis lung carcinoma (Fig. 3).

Uptake of HPD demonstrated by fluorescence

Administration of methylprednisolone acetate (3.0 mg/kg) concurrently with HPD caused a marked reduction in the intensity of HPD fluorescence in Lewis lung carcinoma. Intensity of fluorescence was progressively reduced by increasing doses of methylprednisolone acetate. Hydrocortisone sodium succinate (15 mg/kg) at this time did not cause any alteration in intensity of fluorescence.

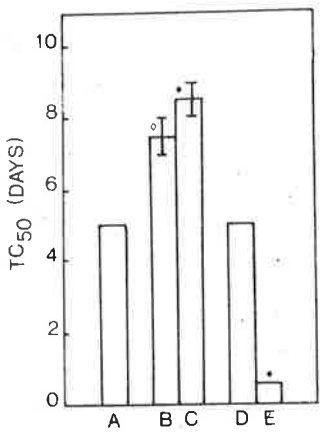

Fig. 4. Interaction between glucocorticoids and PDT of B16 melanoma. Mice with B16 melanoma were given HPD, 30 mg/kg, followed 24 h later by 100 s light to tumours. (A) No glucocorticoid; (B) hydrocortisone sodium succinate, 3.0 mg/kg administered 24 h and 48 h post PDT; (C) hydrocortisone sodium succinate, 15.0 mg/kg administered 24 h and 48 h post PDT; (D) hydrocortisone sodium succinate, 15.0 mg/kg administered with HPD and at time of irradiation; (E) methylprednisolone acetate, 3.0 mg/kg administered with HPD and at time of irradiation. ◇, $P < 0.01$; ◆, $P < 0.001$.

Methylprednisolone acetate (3.0 mg/kg) also caused a reduction in intensity of HPD fluorescence in B16 melanoma while hydrocortisone sodium succinate (15 mg/kg) caused a slight increase in intensity of fluorescence.

Skin photosensitivity

Administration of methylprednisolone acetate had no effect on skin photosensitivity. The mean percentage increase in footpad thickness was 36 ± 20% for mice pretreated with HPD only and 41 ± 14% with HPD plus methylprednisolone acetate.

DISCUSSION

Glucocorticoids administered after PDT with HPD greatly potentiated the therapeutic effect by slowing the rate of recurrence of tumours. By contrast, a high dose of methylprednisolone acetate administered at the same time as injection of HPD strongly inhibited the response to treatment. These effects were observed in two types of tumour. The inhibition of the tumour response by methylprednisolone acetate was dose-dependent. The lowest dose of methylprednisolone acetate examined (0.6 mg/kg) resulted in a prolonged TC_{50}.

Fluorescence of HPD in the tumours decreased with increasing doses of methylprednisolone acetate implying that glucocorticoid inhibited the uptake of HPD. Reduced therapeutic response was associated with reduced intensity of fluorescence.

Both the inhibition of uptake of HPD into tumours by concurrent administration of glucocorticoid and the inhibition of tumour recurrence by administration after an effective treatment with light need to be explained.

Localization of HPD is 5 : 1 in favour of the vascular stroma rather than the tumour cells [4]. Glucocorticoids may inhibit uptake of HPD by decreasing capillary permeability [6,16]. The preservation of the vasculature by glucocorticoids may also protect against photodynamic damage. Henderson [17] showed tumour cells and not killed immediately after irradiation but die at a rate similar to that occurring after vascular occlusion.

Digestion of phagocytosed material may be depressed by hydrocortisone [14]. A proportion of HPD is taken up by phagocytosis [20], so uptake may be depressed by glucocorticoids.

Glucocorticoids are transported by binding to plasma proteins [13] and porphyrin is thought to bind to albumin and haemopexin [11,21]. Competition between HPD and glucocorticoid for binding sites on albumin or other proteins may affect transport of HPD, with unpredictable effects on uptake by tumours.

A primary event in porphyrin induced photodamage may be rupture of lysosomes with release of hydrolytic enzymes [1,5,23,24]. This process may be inhibited by glucocorticoids which stabilise lysosomal membranes [13,27]. However, lysosomal damage may not be a prerequisite for photodamage [22,25].

Administration of glucocorticoids may inhibit repair of photodynamic damage and division by tumour cells. In vitro experiments are in progress to determine if the rate of cell division is directly affected. Repair of the microvasculature may also be retarded by glucocorticoids.

Both the inhibitory effect of glucocorticoids on PDT and inhibition of tumour recurrence may have important implications for the clinical application of PDT. It may be necessary to suspend glucocorticoid treatment for some time before PDT to obtain an adequate tumour response, or alternately use more vigorous treatment conditions during treatment with glucocorticoids. Administration of glucocorticoids after an effective PDT treatment may reduce the likelihood of tumour recurrence. Unfortunately, glucocorticoids had no detectable effect on the skin photosensitivity.

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