Irritant Cutaneous Effects of "Gentian Violet"

A CLINICAL AND EXPERIMENTAL STUDY

Håkan Mobacken

Göteborg 1974

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CONTENTS

This thesis is based on the following publications, which, in the text, will be referred to by their Roman numerals:

I. BJÖRNBERG, A. & MOBACKEN, H. : Necrotic skin reactions caused by 1% gentian violet and brilliant green. Acta Dermatovener (Stockholm) 52: 55 - 60, 1972

II. NORRBY, K. & MOBACKEN, H. : Effect of triphenylmethane dyes (brilliant green, crystal violet, methyl violet) on proliferation in human normal fibroblast-like and established epithelial-like cell lines. Acta Dermatovener (Stockholm) 52: 476 - 483, 1972

III. MOBACKEN, H. & ZEDERFELDT, B. : Influence of a cationic triphenylmethane dye on granulation tissue growth in vivo. An experimental study in rats. Acta Dermatovener (Stockholm) 53: 167 - 172, 1973

IV. MOBACKEN, H. , ZEDERFELDT, B. & ÅHRÉN, CHR. : Effects of two cationic triphenylmethane dyes on the healing of skin incisions. A tensiometric and histologic study in the rat. Acta Dermatovener (Stockholm) 53: 161 - 166, 1973

V. MOBACKEN, H. , AHONEN, J. & ZEDERFELDT, B. : The effect of a cationic triphenylmethane dye (crystal violet) on rabbit granulation tissue: oxygen consumption and RNA and protein synthesis in tissue slices. Acta Dermatovener (Stockholm). In press.

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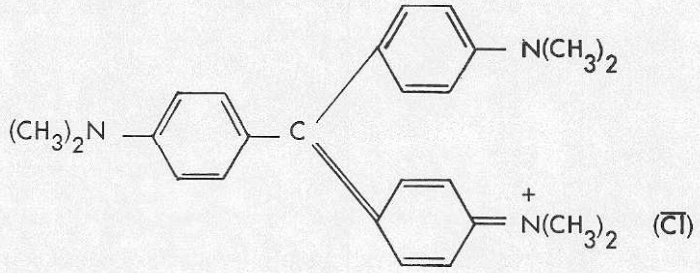
"What is powerful for good
can be potent for evil"

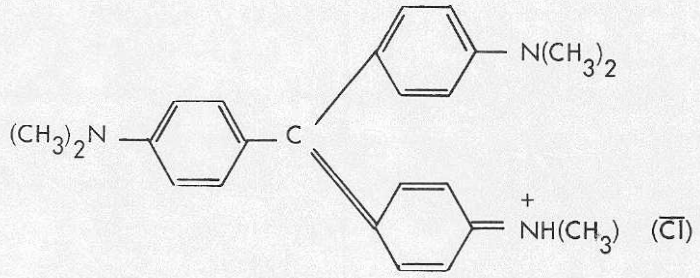
William Boyd, Textbook of Pathology (1961)

Gentian violet and related compounds still occupy an important position in topical therapy because they are powerful and time-honoured antibacterial and antifungal agents. "The triphenylmethane dyes are in Europe among the most used dermatological topical agents" (Bielicky & Novak 1969). Generally gentian violet or crystal violet are preferred. They are usually administered as paints in 0.5 - 2% concentration in aqueous solution. They are used primarily or when newer preparations are not tolerated or are ineffective. The most commonly recommended indications for their use, as stated in standard textbooks and review articles, are: cutaneous and mucosal candidiasis, ringworm infections, primary and secondary bacterial skin infections, and leg ulcers (Pharm. Svec. 1967, DeWeese & Saunders 1968, Brody 1970, Meinhof 1970, Goodman & Gilman 1970, Domonkos 1971, Fitzpatrick et al. 1971, Rook, Wilkinson & Ebling 1972). Such treatment is generally considered harmless.

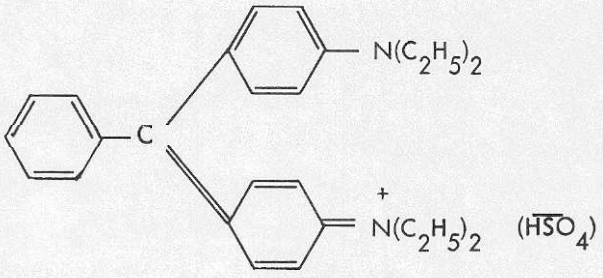
This work was inspired by the clinical observation that some patients treated topically with gentian violet for mycotic infections developed severe irritation and even necrotic ulcers. These reactions could not easily be attributed to the primary or a superimposed infection. Consulted textbooks had no information whatsoever about irritant reactions to gentian violet. A thorough survey of the literature revealed only a few cases of a similar kind and disclosed that little is known about the influence of gentian violet and related compounds on normal and regenerating tissues. It may well be that gentian violet also induces substantial but clinically less dramatic side effects which influence tissue repair.



Hexamethylpararosaniline chloride (crystal violet)



Pentamethylpararosaniline chloride



Brilliant green

Fig. 1. Chemical structure of some triphenylmethane dyes.

Chemical data

The entangled nomenclature of the dyes justifies a short description of those used in this investigation.

Gentian violet, belonging to a group of dyes which are substitution products of the triphenylmethanes, is a mixture of hexa- and penta-methylpararosaniline chlorides (Disp.US 1955, Lillie 1969). The composition is now poorly defined. In fact, various companies may supply different mixtures with this label. Accordingly, the name is no longer considered suitable for a certified stain (Lillie 1969). In clinical practice, however, the name is still retained though the chemist's shops at least in Sweden usually supply hexamethylpararosaniline chloride (crystal violet) when gentian violet is requested (Pharmacop. Nord. 1964).

Crystal violet (C. I. 42555) or methylrosanilinum (Pharm. Nord. 1964) is a pure compound of hexamethylpararosaniline chloride (Merck Index 1960, Lillie 1969). It is a basic dye (pK 9. 4). The pH of the medium affects the degree of ionization of crystal violet (Goldacre & Phillips 1949). In the pH range 6. 5 - 8. 5 the dye is 100% ionized (Fry 1957). Molecular weight 408. Maximum light absorption 589 - 593 nm. At 26°C, its solubility in water is 1. 7% and in alcohol 13. 9% (Lillie 1969). The molecular configuration is depicted in Fig. 1. The benzene rings are not located in the same plane (Buckley & West 1970).

Methyl violet (C. I. 42535) is a mixture of tetra-, penta- and hexamethylpararosaniline chlorides (Lillie 1969). Methyl violet 2B is principally pentamethyl-p-rosaniline chloride (Fig. 1), which has a molecular weight of 394 (Merck Index 1960).

Brilliant green (C. I. 42040) is another basic dye, a diaminotriphenylmethane derivative in the form of the acid sulphate (Fig. 1). Molecular weight 483. Light absorption maximum 628 - 631 nm. (Lillie 1969).

The writer has used crystal violet for the experiments in this thesis (I - V). The commercial product (E. Merck, Darmstadt), pharmaceutical grade, contains not less than 96% of hexamethylpararosaniline chloride and has a small admixture of less methylated derivatives (penta-, tetra-). Methyl violet 2B (II) and brilliant green (I, II, IV) have also been studied.

SURVEY OF THE LITERATURE

Historical background

Churchman, who had investigated the antibacterial properties of the aniline dyes, reported in 1912 that gentian violet has a selective action on most gram-positive bacteria. It is bacteriostatic in dilutions of $1:10^{6}$ in test tube and agar plate, and bactericidal when diluted $1:10^{4-5}$. Support for the rational of a therapeutic usage came from Russel (1914). He found that certain animal tissues were more resistant to the actions of the dye than the bacterial test organism (B. subtilis). Gentian violet was soon tried on patients as a topical antiseptic. In a few patients with severe infections, it was even injected in infected joints and injected intravenously for the treatment of septicemias, with fair results (c. f. Sutton 1938).

In 1920, Churchman emphasized the value of gentian violet in the treatment of wounds infected with gram-positive bacteria. The following years, gentian violet attained widespread use as a topical remedy (c. f. Sutton 1938). Candidiasis soon became another indication for the use of gentian violet (Faber & Dickey 1925), as it inhibits the growth of C. albicans in dilutions of $1:10^{6}$ (Gomez-Vega 1935). The antifungal properties of gentian violet against strains of Epidermophyton, Trichophyton (Gomez-Vega 1935, Geiser 1955) and Microsporon (Geiser 1955) are somewhat weaker than against C. albicans, but sufficiently effective to be employed for the control of superficial dermatomycoses.

There were, however, some studies indicating that gentian violet under experimental conditions might exert harmful effects on tissues. Gentian violet killed rabbit spleen cells in vitro at concentrations not killing S. aureus (Lambert & Meyer 1925-26). At a concentration of 1:800, gentian violet completely inhibited the outgrowth of cells from embryonic chick skin explants (German 1929). Clinicians, however, witnessed the efficacy and safety of gentian violet for topical use. In 1938, Sutton surveyed the literature and his own experiences with gentian violet. He claimed that aqueous solutions of 0.1-2% gentian violet are not irritating, and that he was not aware of any hypersensitive reaction. He concluded: "I have found it valuable in the treatment of monilial infections, especially pruritus vulvae and pruritus ani of this origin, and the cutaneous eruptions dependent on them; of impetigo, infectious eczematoid dermatitis, infantile furunculosis and secondarily infected eczema, infantile or adults; of Vincent´s angina, leg ulcers and ulcers following third degree burns, and of recurrent dermatomycoses including tinea of the hands and feet."

In 1933, Aldrich introduced gentian violet (1%) for the topical treatment of burns, replacing tannic acid. An eschar is formed which seals the burn from contamination, reducing the high frequency of gram-positive infections, and eliminates the loss of fluids through the serous ooze. Following the introduction of this treatment the burn mortality at Johns Hopkins Hospital dropped from 42 to 13% (Aldrich 1937). A combination named "triple dye" of gentian violet, brilliant green and acriflavin eliminates gram-negative bacteria as well (Aldrich 1937). Local treatment of burns with triple dye was continued into the 1940´s (Harkins 1942), when the modern principles of systemic and local burn therapy successively were introduced. At the same time, it was reported that topical burn remedies then commonly employed, including gentian violet and "triple dye", retarded the healing of superficial wounds and burns in man (Cannon & Cope 1943, Dingwall & Andrus 1944) and produced necrosis when painted on exposed rat muscle (Baker 1944). It was also found that gentian violet in concentrations about $1:10^4$ inhibited the phagocytic actions of leucocytes (Welch & Brewer 1942). The findings of these clinical and laboratory studies did not seem to have any obvious impact on the continued widespread

use of the triphenylmethane dyes.

Adverse effects from therapeutic usage

The facts that the underlying disease is masked and that solutions are messy and may stain the patient's clothes and bedlinen are obvious drawbacks of dye treatment.

Sensitization to triphenylmethane dyes has been reported, but is considered to occur very rarely (Rook, Wilkinson & Ebling 1972). There are only a few patients reported with delayed eczematous allergy to gentian violet (Goldstein 1940, Epstein 1958, Bielicky & Novak 1969). Cross-sensitization may exist between chemically related triphenylmethane dyes (Bielicky & Novak 1969).

Immediate hypersensitivity reactions (anaphylactic chock) have occurred due to sensitization to topically applied methyl green (which is crystal violet with an extra ethyl group) (Francois et al. 1971).

Irritant reactions have also been observed. Since the introduction of indelible pencils more than 70 years ago, ophtalmologists and surgeons have been aware of the risk for tissue necrosis following accidents to the eyes or skin (c. f. Strandell 1950). The causative agent is considered to be gentian violet, which may be present in a concentration as high as 30% (Hosford & Smith 1952). "Epidemic" nosebleed in apple packers was caused by dust released from apple-packing trays coloured with gentian violet (Quinby 1968). Slotkowsky (1957, 1966) reported two infants out of several with thrush, who developed tender ulcerative or gelatinous-like lesions of oral mucosa following painting with 1% aqueous gentian violet for 7 weeks and 6 days. He was also able to produce similar lesions in rabbit oral mucosa by application of 1% aqueous solution of gentian violet for 2 weeks. In 1968, John reported 2 infants treated 1 and 2 weeks with 0. 5% gentian violet for oral candidiasis who developed necrotic ulcerations of the oral mucosa.

Jennison & Llywelyn-Jones (1957) treated 36 patients suffering from candidal vaginitis with 1% aqueous gentian violet on three alternate days. Six of the women developed a local reaction of the vagina which made it necessary to stop treatment. Clabaugh (1968) has described a method to remove tattoo-marks, utilizing superficial dermabrasion followed by daily paintings with 2% aqueous solution of gentian violet. The reason for using the dye is not specified but it appears that it was intended to serve as an antiseptic. This procedure may result in a cutaneous necrotic inflammatory reaction (Hersle 1970). Sutton (1938) reported 2 cases of scar tattooing with a purple colour resulting from contact of gentian violet with granulation tissue.

Gentian violet taken orally as a vermifuge may cause diarrhoea, nausea and vomiting, suggesting a local effect on the gastrointestinal mucosa (Beckman 1961, Martindale 1967).

The aims of the present investigation of some cationic triphenylmethane dyes were to study:

1) the clinical picture of lesions caused by such dyes in man (I),

2) the influence of epicutaneous dye application on normal skin and on skin with a disturbed penetration barrier ("stripped" epidermis) (I),

3) the effect on cellular proliferation in vitro (II),

4) the effect on the formation of granulation tissue[x] and on the rate of wound healing (III, IV),

5) the early metabolic reactions of granulation tissue following dye exposure (V).

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[x] Granulation tissue: a newly formed, highly vascularized connective tissue with a component of acute inflammatory exudation (Cameron 1969).

CLINICAL PICTURE OF LESIONS CAUSED BY TRIPHENYLMETHANE DYES IN MAN (I)

Three patients, out of several, are described who developed necrotic lesions after the application of 0. 5-1% gentian violet in aqueous solution to the skin of the scrotum, the napkin area of an infant and the vulva.

The first sign of an untoward reaction in the patients was the appearance after up to 10 days of intensely painful, somewhat swollen spots. A slight exudation occurred, followed by sloughing of tissue and resulting in superficial and very tender ulcerations with a characteristic yellow (sometimes black) surface. In intertriginous locations, the ulcers sometimes merged to form large, denuded areas. Rapid healing followed cessation of gentian violet treatment.

Patch tests were performed on 2 of the patients on normal back skin with 1% gentian violet (the bottle, delivered from the hospital pharmacy, was labelled "gentian violet", but actually contained crystal violet) and 1% brilliant green in water. The patch tests showed negative results.

Conclusion. Necrotic ulcerations may occur on injured skin painted with 1% gentian violet in aqueous solution.

EPICUTANEOUS DYE APPLICATION ON NORMAL AND "STRIPPED" EPIDERMIS (I)

Partial epidermal defects were made by stripping away the stratum corneum with adhesive tape, as originally described by Pinkus 1951 (for review, see Christophers 1972). Two human volunteers and 10 guinea pigs were used for these experiments. In man, immediately after stripping on the flexor surface of the forearm, 1% gentian violet (crystal violet, vide

supra) in water was painted once a day for 2 days on half of the stripped area, the other half serving as control. Identical experiments were done with 1% brilliant green (aq. sol.). The guinea pigs were stripped on the flank and immediately painted, 5 animals with 2% gentian violet (crystal violet, vide supra) and 5 with 2% brilliant green. Skin biopsies were taken from stripped areas after 1 and 5 days in man, and after 3 days in guinea pigs.

Application of dyes to the stripped skin of man and guinea pig gave essentially the same result. A sequence of gross skin changes almost identical to that described in the patients occurred. All guinea pigs had developed necrotic reactions after 2 days, but only in painted areas that had previously been stripped. In man, ulcerations were seen after 5 days in areas painted with gentian violet as well as brilliant green. Application of these dyes to the intact skin of guinea pig or man (Björnberg & Mobacken 1972) produced no reaction.

In man, hydropic degeneration of the basal layer with subepidermal vesicle formation and a polymorphonuclear leucocytic infiltration in the upper dermis was found in stripped areas 24 hours after the first dye application. After 5 days, there was total epidermal necrolysis. In guinea pigs, most epidermis was lost after 72 hours, and in some places subepidermal vesicles were seen. Small leucocytic and lymphocytic infiltrates appeared in the dermis.

Conclusion. Reactions similar to those seen in clinical practice can be reproduced by painting "stripped" - but not "unstripped" - normal skin in man and guinea pig.

TRIPHENYLMETHANE DYES AND CELLULAR PROLIFERATION IN VITRO (II)

In monolayer cultures, one fibroblast-like cell line from human dermis was exposed to crystal violet, brilliant green and methyl violet 2B at concentrations of 1-100 µg/ml ($1:10^6$ - $1:10^4$) for 3 days. Epithelial-like cells of human origin (HeLa-cells) were similarly exposed to crystal violet. Single-cell suspensions of cultures were electronically analysed for cell-counts. For subsequent studies, the skin fibroblast-like cell line and crystal violet at a concentration of 10 µg/ml were chosen. In one series of experiments the cell number of cultures continuously exposed to this dye was measured 24, 48, 76 and 96 hours after subculture. In another experiment, crystal violet was added to dense, topo-inhibited, somewhat tissue-like 12-days-old cultures, which were analysed 2 days later. In a third series of experiments, crystal violet was added to cells in growth media containing heparin (conc. 0.1-200 IU/ml), chondroitin sulphate (100 µg/ml), Na-dextran sulphate (100 µg/ml) or varying concentrations of serum (2.5, 10 and 40% v/v). The cells were counted 3 days later. In the last experiment the effect of various durations of exposure to crystal violet (5, 20, 80, 320, 1280 minutes) was tested by small inocula of cells and counting the number of multicellular colonies 10 days later.

Three days' exposure to all dyes markedly reduced the number of cells. The most pronounced effect regularly occurred at an intermediate concentration (10 µg/ml). The reduction in cell number was progressive, being substantial already after 24 hours. The addition of crystal violet to 12-days-old cultures caused a marked decrease of cells in the originally dense cultures. When the medium contained 40% serum or when the polyanionic substances heparin, chondroitin sulphate or Na-dextran sulphate were added to the growth medium, the cytotoxic effect of crystal violet was reduced. Already 5 minutes' exposure to crystal violet resulted in a significant reduction of the number of cell colonies. Increasing exposure times resulted in further decrease of cell colonies.

Conclusions. Crystal violet, methyl violet 2B and brilliant green were strongly cytotoxic in vitro at concentrations as low as 1 µg/ml ($1:10^6$). Exposure to crystal violet (10 µg/ml) for 5 minutes was enough to hamper the multiplication of cells. The toxicity of crystal violet was pronounced in densely as well as in sparsely populated cultures. The cytotoxic actions of crystal violet were markedly reduced by high serum concentration and polyanions such as heparin and chondroitin sulphate.

TRIPHENYLMETHANE DYES AND CONNECTIVE TISSUE REGENERATION

This problem was evaluated in two different models.

Subcutaneous sponge implantation (III)

Viscose cellulose sponges were used as an inductive matrix for granulation tissue, as described by Viljanto (1964). Each sponge was cut as a cylinder and sectioned into 2 equal halves (each 1 x 1 x 1 cm), which were sewn together in their original position. One anterior and one posterior incision was made under sterile conditions in the dorsal midline of male rats. Through each incision one sponge was implanted subcutaneously on each side. In 4 groups of animals, one sponge in each pair was exposed immediately before implantation to an aqueous solution of crystal violet and the other to sterile water. The dye was used in dilutions of 1:100, 1:1,000, 1:2,000 and 1:10,000. In a fifth group, the subcutaneous wound pockets were correspondingly treated with crystal violet (1:2,000) for 5 minutes and the sponges soaked in sterile water.

Ten days after implantation, the sponges were removed and carefully freed from surrounding tissue. The suture holding the halves of the implant together was removed. The breaking load of the newly formed tissue connecting the 2 halves of each sponge was measured with a tensiometer. The dry weight of the tissue within the sponge was determined. Paired comparisons were used for each pair of sponges. In additional animals, similarly treated, implanted sponges were used for histology.

Exposure of the tissues either by dyeing the sponge or the wound pocket resulted in a marked decrease of the strength of the tissue bridging the narrow cleft between the 2 halves of each sponge. The dry weight of tissue contained in the sponge was significantly reduced in sponges treated with crystal violet 1:10, 000, and in sponges implanted in a dye-treated wound pocket. Histological examination disclosed considerable ingrowth of connective tissue into control sponges. Dye-exposed sponges, irrespective of dye concentration, seemed to contain no connective tissue at all, the sponge pores being occupied by serofibrinous oedema and inflammatory cells. The sponges were not serially sectioned. Therefore the occurrence of some connective tissue at least in sponges least exposed to crystal violet cannot be excluded. Necrotic areas on skin overlying a dye-soaked sponge were observed in 4 rats out of the 10 treated with the highest concentration of crystal violet, 1:100.

Conclusion. Crystal violet in concentrations well below those used clinically markedly inhibited the formation of granulation tissue in cellulose sponges implanted subcutaneously for 10 days.

Wound healing (IV)

Two standardized skin incisions were inflicted symmetrically on the back of rats. The incisions, 6 cm long, were perpendicular to the skin surface and penetrated the subcutaneous muscle. One wound was exposed for 5 minutes to an aqueous dye solution and the other to sterile water. This duration had experimentally been found sufficient to interfere with cell multiplication in vitro (II). The incisions were closed by continuous silk suture, the wound edges being carefully adapted. Crystal violet was used in dilutions of 1:1, 000, 1:2, 000, 1:10, 000, 1:20, 000, and 1:50, 000. Brilliant green was diluted 1:2, 000. After 7 days the sutures were removed and the breaking load of each wound was determined in situ with a tensiometer. The percentage difference between dye-exposed and control wound in each rat was calculated and used for statistical analysis. Inter-individual variation was avoided by comparing control and test wounds in the same animal.

In another experiment, groups of animals were examined for wound strength after healing periods of 7, 10, 14 and 21 days after exposure to crystal violet 1:2, 000. In additional animals, wound biopsies were taken for histology. For each animal coded slides with specimens from the test and the control wound were compared. The degree of the inflammatory reaction, the proliferation of fibroblasts, the formation and structure of collagen fibres and the formation of ground substance were evaluated.

Wounds treated with brilliant green or with all tested concentrations of crystal violet had significantly reduced values for breaking load 7 days postoperatively. The results from the animals treated with crystal violet 1:2, 000 and followed for 3 weeks demonstrated a lengthened lag phase and consequently a delay of the onset of the period of fibroplasia. Once started, however, the rate of gain of breaking load was almost as rapid in dye-treated as in control wounds. At 3 weeks postoperatively, the test wounds had still not reached control wound values.

Histologically, the dye-treated wounds exhibited an intensified and prolonged acute inflammatory reaction. After 3 weeks´ healing, the amount of fibroblasts and collagen seemed roughly equal in wounds treated with dye and water. There were, however, qualitative differences: in dye-treated wounds, the fibroblasts and the collagen fibres were less mature.

Conclusions. One application of crystal violet to incisional skin wounds before suturing retarded the development of strength in the wounds for at least 3 weeks. The dye prolongs the lag phase by inducing an increased and prolonged acute inflammatory reaction, thus retarding the onset of fibroblast proliferation and collagen formation.

EARLY METABOLIC REACTIONS OF DYE-EXPOSED GRANULATION TISSUE (V)

Albino rabbits were used as donors of granulation tissue, obtained from stainless steel wire mesh cylinders implanted subcutaneously 3 weeks previously according to Holmström (1973). The tissue within the cylinder was placed in an ice-cold buffered incubation medium, sliced, weighed and divided in samples about 500 mg. Experimental samples were exposed to crystal violet 10 µg/ml ($1:10^5$) or 1 µg/ml ($1:10^6$) (dissolved in the incubation medium) for 5 minutes at 37°C, and control samples correspondingly treated with the incubation medium. The determination of oxygen uptake of tissue samples started either immediately after exposure, or 30 minutes later. The method was a slight modification of that recently described by Holmström and co-workers (1974). The incorporation of ^{14}C-proline into proteins and collagen hydroxyproline was estimated by incubation with 5 µCi L-proline-^{14}C(U) at 37°C for 2 1/2 hours. The RNA-synthesis was determined following exposure of tissues to 50 µCi cytidine-5-^{3}H at 37°C for 2 1/2 hours and isolation of RNA. Incorporation of ^{3}H-cytidine into RNA was taken as a measure of newly formed RNA.

Exposure of sliced granulation tissue to crystal violet $1:10^5$ for 5 minutes caused a markedly reduced incorporation of ^{14}C-proline into proteins as well as into collagen hydroxyproline. The synthesis of RNA was clearly reduced. The oxygen consumption rate was only slightly reduced as compared to control samples when the assay started immediately after exposure to the dye, but markedly lower in samples analysed 30 minutes later. With crystal violet $1:10^6$, the incorporation of ^{14}C-proline into proteins and collagen hydroxyproline was reduced, but with this concentration of dye the difference from control samples was less. The synthesis of RNA and the oxygen consumption rate (assay starting 30 min. after exposure) were not affected.

Conclusions. Granulation tissue slices exposed in vitro to crystal violet in a dilution of $1:10^5$ has a decreased capacity to consume oxygen, to incorporate ^{14}C-proline into collagen and non-collagenous proteins and to synthesize RNA.

Gentian violet has been used as a topical antibacterial and antifungal agent for more than half a century and is still widely used (Pharm. Svec. 1967, Domonkos 1971, Rook, Wilkinson & Ebling 1972). Only a few reports of adverse effects of this remedy are available (see References in I) and textbooks of dermatology regularly consider this treatment as having very few complications (e. g. Hellerström 1968, Domonkos 1971, Fitzpatrick et al. 1971, Rook, Wilkinson & Ebling 1972). Except for some few experimental studies showing a cytotoxic effect of gentian violet (Lambert & Meyer 1925-26, German 1929, Welch & Brewer 1942) little is known about the influence of this substance on living cells. The exact incidence of tissue damage from cationic triphenylmethane dyes can only be discovered from well controlled, prospective studies. Search of the literature has only revealed one such study (Jennison & Llywelyn-Jones 1957). These authors found, in a clinical trial of nystatin for candidal vaginitis, that out of a control group of 36 women treated with 1% aqueous gentian violet no less than 6 developed severe local reaction which necessitated cessation of treatment. Though this frequency appears high it points to the possibility that a considerable number of cases with adverse tissue reactions to triphenylmethane dyes are not recognized or not reported. Incomplete knowledge of the influence of dyes on normal and reparative tissue increases the risk of a misinterpretation of the clinical picture. Besides, the "index of suspicion" for unexpected adverse drug reactions is low with time-honoured remedies. The clinical observation of marked tissue reaction in some patients treated with gentian violet motivated analyses with different experimental models of the possible tissue damaging effects of chemical compounds of this type.

The standard method for screening and comparing the potential cutaneous irritancy of drugs and cosmetics is that described by Draize and coworkers (1944) using rabbits. A critical review of this method has been given by Rostenberg (1961). The method is unsuitable for studies of

coloured compounds since the tests are numerically scored by the degree of erythema and oedema. A simple method for studying coloured compounds is to apply the dye to intact and "stripped" (i. e. stratum corneum removed) guinea pig skin. This method has been used in the present investigation. It was found (I) that crystal violet caused marked gross and microscopical changes when the dye was applied to "stripped" skin, thus tissue where the protective barrier has been removed. The microscopical changes were distinct and comprised necrosis of epidermal cells, epidermo-dermal separation and dermal polymorphonuclear leucocytic infiltration. These features are characteristic of a primary irritant dermatitis (Hunziker 1969, Medenica & Rostenberg 1971). No reaction was observed when the dye was applied to skin with intact stratum corneum.

The reactivity of guinea pig skin to irritants approximates that of human skin (Davies, Harper & Kynoch 1972). Application of crystal violet to skin in man using the same technique as described for guinea pigs resulted in similar gross and microscopical changes.

The observations (I) indicate that application of crystal violet under certain conditions can cause serious tissue damage. Such conditions can be expected to occur in most instances of clinical use where infectious and inflammatory situations are at hand. The observations further indicate that the possibilities for penetration of the applied dye into the tissue are of importance. Penetration is known to increase with occlusion (c. f. Wahlberg 1973) - from dressings or natural body folds - and consequently an increased risk for tissue damage will exist in such situations.

After the clinical and experimental observations of a tissue damaging effect of gentian violet this effect was studied more in detail by methods that allow quantitation. It was decided to study the influence of dye on regenerating tissue, because this would closely approximate the clinical situation in that the process of regeneration involves phases of inflammation and fibroplasia.

Two methods, subcutaneous implantation of cellulose sponges and breaking

load determinations of incised skin wounds, were used for study of the regeneration of connective tissue. Both methods are well defined and allow quantitative determinations of the influence of locally applied agents (Sandblom 1944, Viljanto 1964, Rydberg 1968, Brunius 1968). The technique of cellulose sponge implantation (III) demonstrated that exposure of the sponges or the tissue around the sponges to crystal violet resulted in increased inflammatory reaction and marked reduction of tissue formation, illustrated as persistent serofibrinous oedema in sponges and decreased strength of a defined tissue bridge in the sponge. These experiments thus confirmed and quantitated a tissue damaging effect of crystal violet. Further support for this negative influence was found in the study of breaking load development in sutured skin wounds (IV) which demonstrated significant reduction in breaking load after local application to the wound edges of the dye. Microscopic study of the wounds revealed a prolonged and intensified inflammatory reaction. The study of the development of wound strength over the period 7 - 21 days showed that this retardation of healing was due mainly to a prolongation of the lag phase while fibroplasia, once started, proceeded at a fairly normal rate. However, breaking load was still at 21 days lower in dye-exposed wounds indicating that up to that time the delay in healing had not been compensated. These results, obtained from wounds healing by primary intention, should be valid also for open wounds (healing by secondary intention), as the principles of the healing process are the same (Schilling 1968). The observations thus support the findings by Cannon & Cope (1943) and Dingwall & Andrus (1944) of retarded healing of abraded or burned skin treated with gentian violet.

Some degree of inflammatory reaction is required for optimal healing. Decreased as well as increased inflammation will cause impaired healing (Carrel 1921). The degree of inflammation optimal to healing is not defined but empirically the most appropiate stimulus is "the clean sweep of a sharp cold knife" (Dunphy 1963). Merely the presence of sutures in such an incisional wound will add to the inflammation and impair healing (Brunius 1968), but at the same time has the positive effect of allowing primary healing. These mixed effects of a local factor can of course also apply to the use of crystal violet: the negative effect of tissue damage and the

positive antimicrobial effect. Observations similar to those in the present study have been reported by Rydberg (1968) as regards quaternary ammonium compounds with antibacterial effects.

The tissue damage observed by the different experimental methods is the end result of tissue reaction to the dye. Elucidation of the mechanisms behind the dye impairment of tissue function required more sophisticated methods, such as tissue cultivation and biochemical analyses of regenerating tissue.

The study of the influence of crystal violet on human cells in vitro (II) demonstrated that a short application of only 5 minutes already causes marked impairment of cell multiplication and also that, with longer exposure, this impairment is increasingly pronounced. From the study it can further be inferred that the cytotoxic effect can be partly reduced by the addition to the medium of high concentrations of serum or polyanionic tissue components. The marked cytotoxicity of crystal violet even in cultures with a reduced growth fraction indicates that the dye damages not only DNA-synthesizing cells but apparently also cells in other phases of the cell cycle. It might well be that crystal violet interferes with basic mechanisms of living cells.

In a further attempt to analyse crystal violet´s mechanism of action (V), granulating tissue exposed to the dye was analysed biochemically and functionally by measurements in vitro of the oxygen consumption capacity. It was observed that exposure to crystal violet for 5 minutes resulted in decreased oxygen consumption, being more marked with time after exposure. Reasonable explanations can be either a disturbance of the energy metabolism dominant in these cells (glucose intermediary metabolism and mitochondrial function) or an adaptation to less energy demands resulting from decreased cytoplasmic protein synthesis as evidenced by the reduced incorporation of ^{14}C-proline into collagen and non-collagenous proteins. The synthesis of RNA was also reduced. It was thus possible to analyse in somewhat greater detail the immediate modifications of the cellular biochemical processes induced by crystal violet, which finally will result in

gross tissue damage. It must be observed, however, that further work is needed to explore the primary effect of the dye on specific cellular constituents. Although the body of evidence seems to support a direct cell influence it must be cautioned that the results obtained could partly be due to an influence on the intercellular ground substance leading to a decreasing passage of nutrients to the cell and changing the milieu for aggregation of collagen fibrils (c. f. Preston & Snowden 1973).

A cytotoxic effect of crystal violet on exposed tissue seems thus to be clearly demonstrated in these experimental models. A few parallel experiments have been done with related compounds, which turned out to be generally equivalent as regard their tissue damaging effect. The dyes tested were brilliant green (I, II, IV), methyl violet 2B (II) and basic fuchsin (Björnberg & Mobacken 1972).

Theoretically, there is a risk that the decreased tissue viability inflicted by a triphenylmethane dye will make the traumatized tissue more infectable (Burke 1964), e. g. from surviving gram-negative bacteria. This point deserves further study, as a recent increase in Pseudomonas infections has been reported in dermatologic practice (Noble & Savin 1971).

The experimental studies thus demonstrate that crystal violet and related triphenylmethane dyes in concentrations well below those used in clinical practice under certain circumstances can induce considerable tissue damage. Certainly different species of animals were used in the experiments but the healing process, being a basic biological function, is considered not to differ qualitatively between them and man (Howes, Harvey & Hewitt 1939). This is corroborated by the congruent results in humans and guinea pigs (I). Consequently, it seems reasonable to assume, that the results of the experimental studies should in principle be valid for humans too. Considering the widespread use of crystal violet and its irritant potency, it remains to be explained why tissue damage is not more frequently reported in the literature or observed in practical work. The degree of tissue damage will be influenced by several factors. Such factors of obvious importance are: the concentration of the dye and exposure time (I, II),

the concentration of dye neutralizing substances such as serum and polyanionic tissue components (II), the condition of the treated tissue (I) and the occurrence of occlusion (c. f. Wahlberg 1973). Regional variations in percutaneous penetration of various compounds exist between different body regions (Cronin & Stoughton 1962, Winkelmann 1969); the scrotal skin being particularly penetrable (Smith, Fischer & Blank 1961, Wahlberg 1973). Besides, some individuals seem to have a constitutionally increased sensitivity to certain primary irritants[x] according to clinical and experimental findings (Kligman 1963, Björnberg 1968). A generalized, unspecific increase in skin reactivity ("status eczematicus") can temporarily arise as a consequence of localized eczemas, rendering not obviously affected skin more susceptible to primary irritants (Björnberg 1968), and add to the risk of unexpected side effects from therapeutic agents.

The demonstration of the tissue-damaging effect of the triphenylmethane dyes will, when added to other disadvantages such as discolouring and masking, probably make them clinically less common. However, this should not preclude their use, as long as one is aware of the possible injurious effect and precautions are taken to reduce this risk.

The experimental models used in this investigation may be useful for the study of tissue-damaging effects of other topical drugs. Exploration of the possible tissue-irritating effects along the lines presented here of new chemicals intended for clinical use may be helpful in revealing even weak irritants and create opportunities for taking adequate precautions before their use in humans.

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[x] Primary irritants are substances which damage skin by direct action (Kligman & Wooding 1967).

The purpose of the present investigation was to study the influence of triphenylmethane dyes on normal and reparative tissue. The impetus was the clinical observation (I) that necrotic ulcerations sometimes develop in treated areas, a little known complication. It was possible to reproduce the necrotic reaction in man and guinea pig by painting an aqueous solution of crystal violet on skin where the stratum corneum had been stripped away with adhesive tape (I). "Unstripped" skin showed no reaction, which indicates a poor penetrability into normal skin.

The tissue-damaging effect was further studied by implanting viscose cellulose sponges subcutaneously in rats (III). Exposure of the sponge or the surrounding tissue to crystal violet resulted in a pronounced reduction of the breaking load over a defined tissue bridge in the sponge. Microscopically, the sponges contained a serofibrinous oedema with inflammatory cells and appeared devoid of newly formed connective tissue.

The significance of this observation for wound healing was evaluated by exposing incisional wounds in rat skin to the dye for 5 minutes (IV). Healing, as measured by breaking load, was still retarded after 21 days. The lag phase was prolonged due to an intensified and lengthened post-traumatic inflammatory reaction. This delayed the onset of fibroplasia, but, once started, it proceeded at a fairly normal rate. Morphologically there was evidence of slower maturation of granulation tissue. The results are probably valid also for open wounds.

The mechanism behind the deleterious tissue effect of crystal violet and related triphenylmethane dyes was studied by a tissue-culture technique (II). The dyes were strongly cytotoxic against fibroblast-like cells and HeLa-cells. Exposure for 5 minutes was sufficient to hamper the multiplication of cells; increasing exposure times produced further decrease of the number of cell colonies. High concentrations of serum and

polyanionic tissue components (heparin, chondroitin sulphate) partly reduced the cytotoxic effect. These substances may constitute a tissue protective factor when triphenylmethane dyes are used clinically. The cytotoxicity was not reduced in dense, topo-inhibited cultures as compared with vigorously proliferating cell populations, suggesting that crystal violet affects basic cell functions and not only DNA-synthesizing or dividing cells. This was analysed in greater detail in granulation tissue by measuring the capacities to consume oxygen, to incorporate ^{14}C-proline into collagen and non-collagenous proteins and to synthesize RNA (V). These processes were markedly reduced by crystal violet.

The experimental models demonstrate a tissue-damaging capacity of triphenylmethane dyes in concentrations well below those used clinically. This fact must be paid attention to when therapeutic alternatives are discussed in clinical practice. It should not preclude their use, however, as long as one is aware of the possible injurious effect and take precautions to reduce this risk.

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