Istituto Nazionale Tumori, Milano

Mechanism of Action of Daunomycin*

A. Di Marco

Daunomycin is a metabolite of *Streptomyces peucetius* (Grein *et al.*, 1963) which strongly inhibits a variety of experimental tumors (Di Marco *et al.*, 1964).

This substance is a glucoside (Clorhydrate: $C_{27}H_{29}NO_{10}$ HCl) which yields by acid hydrolysis an aglycone, daunomycinone (Fig. 1) (Arcamone *et al.*, 1964).

This antibiotic shows a strict relationship with anthracyclines: in this group should be included, following the Brockmann suggestion (Brockmann, 1963) those antibiotics containing the tetrahydrotetracenquinone cromophore linked to a sugar.

Activity on *in vitro* grown normal and neoplastic mammalian cells

Da has a strong inhibiting effect on the *in vitro* growth of mammalian cells; a 0.1 μg/ml dose on slide cultures of rat fibroblasts and Hela cells reduces considerably the mitotic activity (Fig. 2) (Di Marco *et al.*, 1963).

Comparable activity was shown (Di Marco *et al.*, 1963) on cultures of cells originally isolated from carcinoma of the larinx (Helius Lettré strain) and from human epidermoid carcinoma (KB strain). In *in vitro* mouse and rat bone marrow, both in rotating tubes and hanging drop cultures added with 0.1 μg/ml of Da, absence of mitoses and severe cytological damages were observed after 24 hours (Di Marco *et al.*, 1963). Da, at a concentration of 1.0 μg/ml, was found to inhibit the proliferative activity of stem cells developed from human lymphocytes, following phytohemoagglutinin stimulation (Costa and Astaldi, 1964).

The flourescence of Da permits to show the penetration of this substance into the cell and the fixation in correspondence of the nuclear structures (Fig. 3).

Cell damage induced by Da is mainly nuclear: in resting cells a finely granular appearance of the chromatin and marked alteration in the shape and size of the nucleolus have been remarked (Figs. 4-5).

Cytoplasmic changes, such as vacuolization, are moderate and appear later and only after prolonged treatment by high doses. In mitotic cells chromosomal damage such as fragmentations and mitotic aberrations, mainly anaphasic bridges, were observed (Figs. 6-7-8-9) (Di Marco *et al.*, 1963). The reduction in mitotic index, after addition of Da, appears rather abruptly (Fig. 10). If a sufficient amount of the anti-

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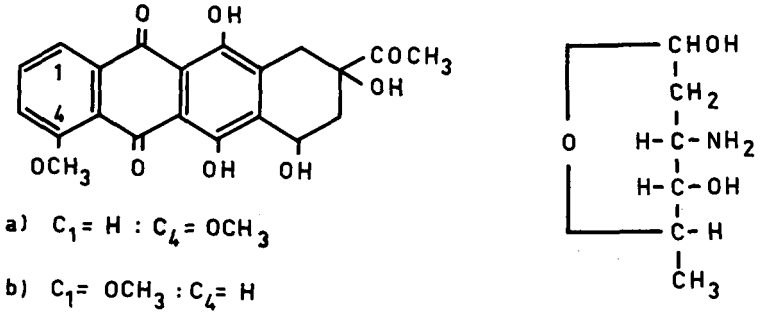

Fig. 1. Chemical structure of Daunomycin

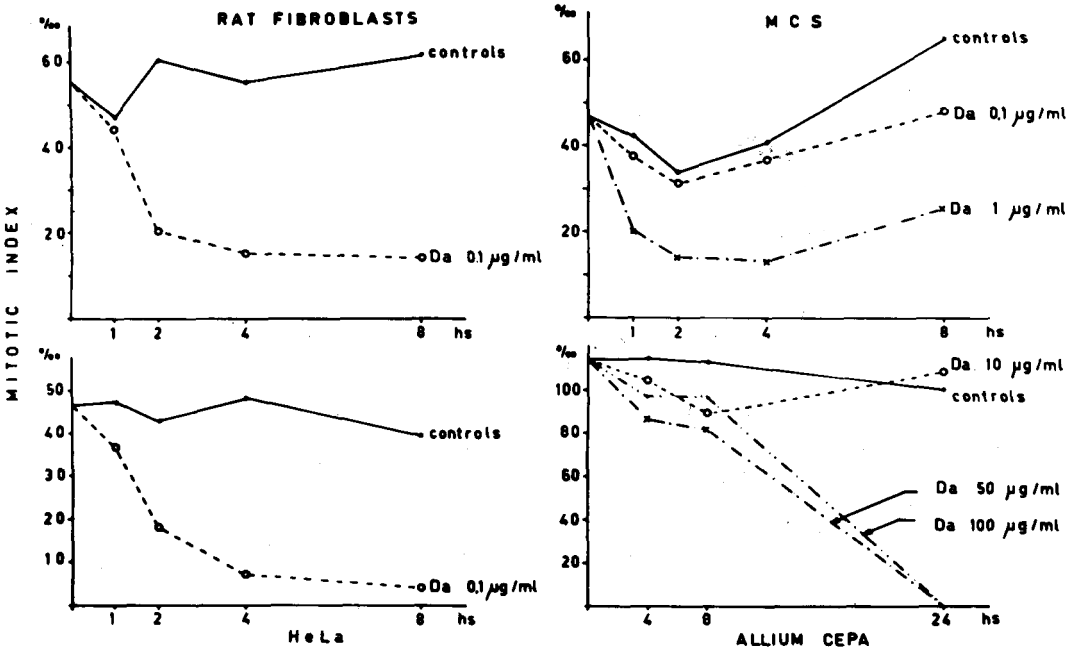

Fig. 2. Action of Daunomycin on cultured cells

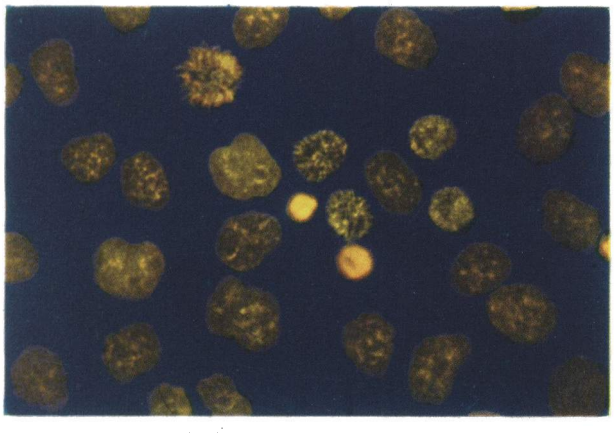

Fig. 3. Evidence of the presence of Daunomycin 5 µg/ml in Hela cells at the fluorescent microscopy (incubation 30' at the 37° C)

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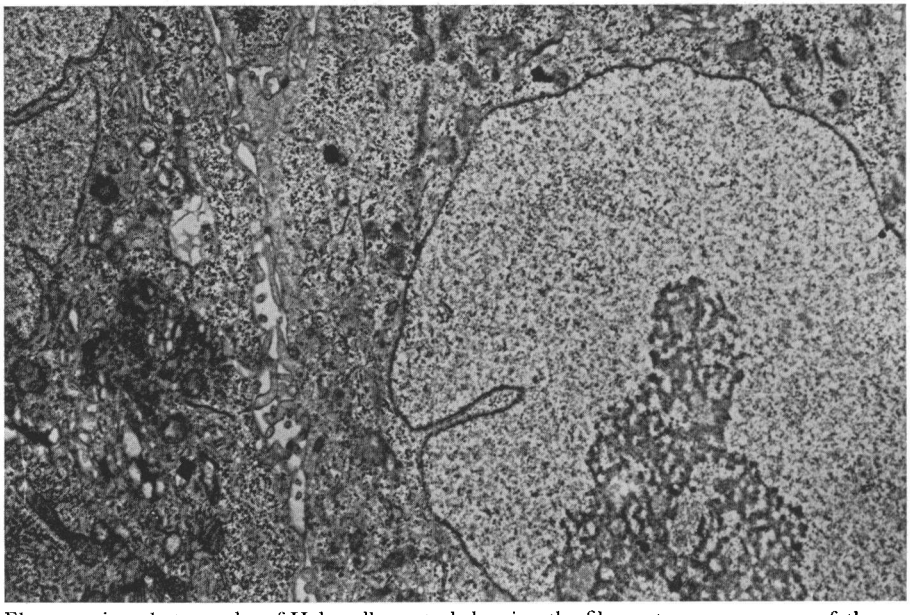

Fig. 4. Electronmicrophotography of Hela cells control showing the filamentous appearance of the nucleolus. Many RNA granules adherent to the skeinlike network of the nucleolonema are evident

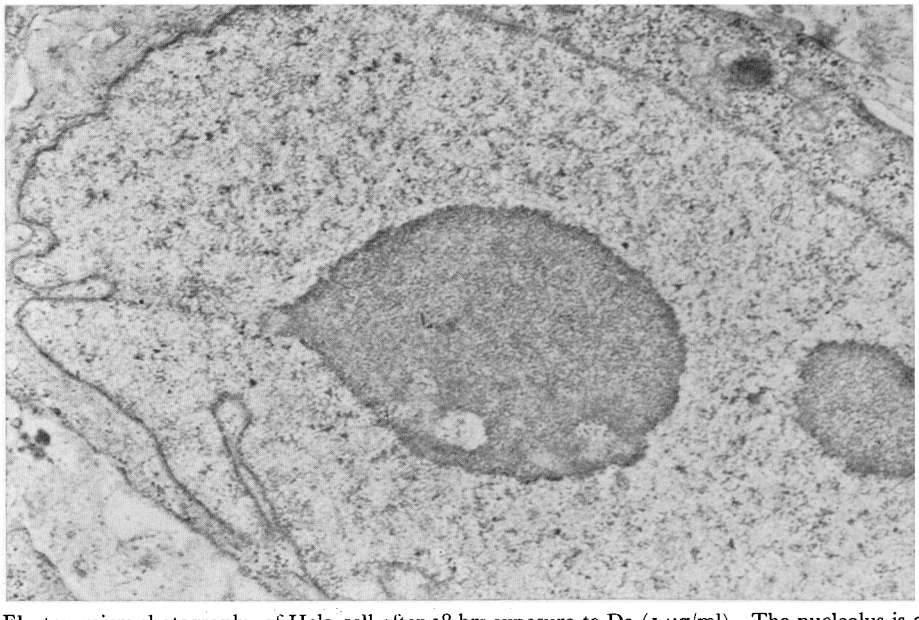

Fig. 5. Electronmicrophotography of Hela cell after 18 hrs exposure to Da (1 μg/ml). The nucleolus is changed into a dense and compact body showing large areas in which RNA granules have completely disappeared

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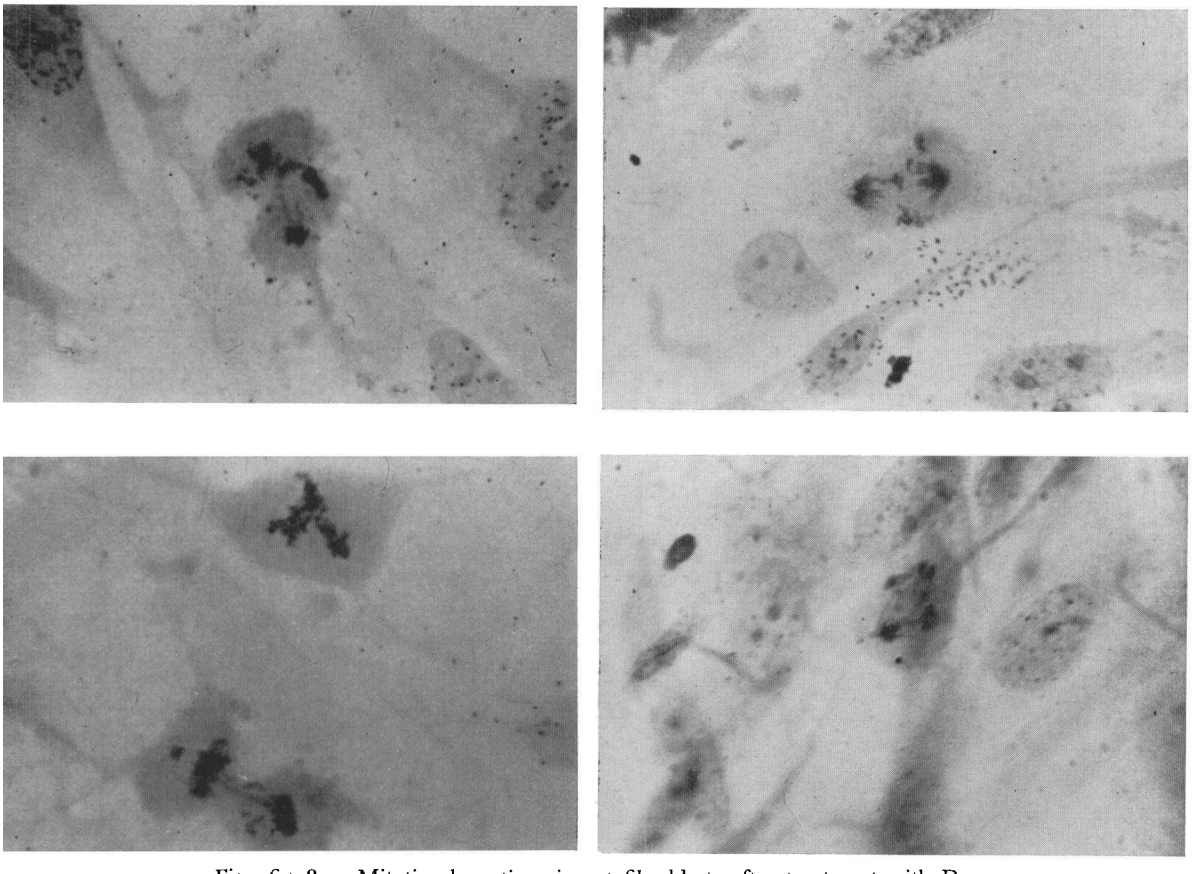

Figs. 6-7-8-9. Mitotic aberrations in rat fibroblasts after treatment with Da

biotic is added to a rat fibroblasts culture it is possible to observe, by phase contrast microscopy, the blocking of the mitotic process and an anomalous scattering of the chromosomes within the cell (Di Marco *et al.*, 1963).

In cultured mammalian cells Da strongly inhibits 8-^{14}C adenine incorporation into RNA (Rusconi and Calendi, 1966).

Autoradiographic researches carried out on *in vitro* cultures of Hela cells (Di Marco *et al.*, 1965) showed a different degree of susceptibility to Da of ^{3}H uridine incorporation into RNA which can be observed at the nucleolar and extranucleolar level (Figs. 11-12-13-14).

These observations confirm the previous studies of Perry (1963) on the higher susceptibility to Actinomycin D of the ^{3}H uridine incorporation in the nucleolar area as compared to that occurring in the extra-nucleolar " chromosomal " area.

Therefore, trials were made to identify by physico-chemical procedures the RNA

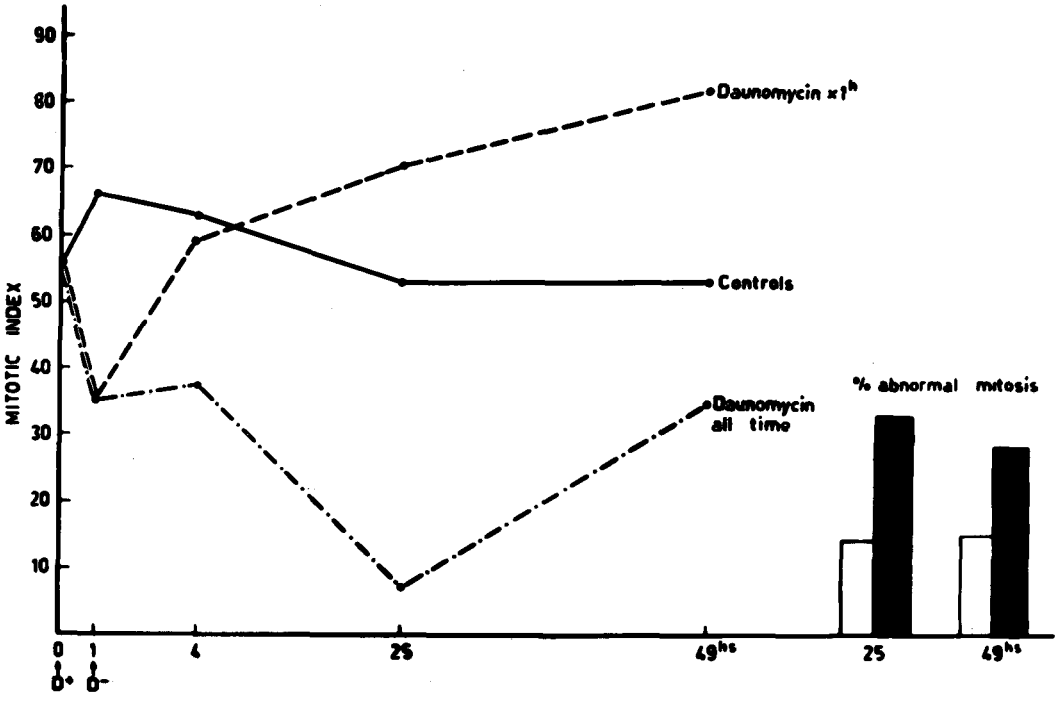

Fig. 10. Action of Daunomycin on Hela cells monolayer

fraction whose synthesis is differently inhibited by these antibiotics. Experiences carried out on Hela cells demonstrated Da to have a considerable degree of inhibition on newly synthesized RNA (extracted by phenol-sodium-dodecyl-sulphate method, according to Hiatt, 1962) which sediments in sucrose gradient in the zone 30 S and 20 S (Figs. 15-16) (Rusconi and Calendi, 1966).

On the contrary an RNA aliquot not extracted by the indicated procedure appeared less sensitive to the action of Da and Actinomycin. At the present stage of the research it would be premature to identify this RNA fraction with " chromosomal RNA " however, reference should be made to the published reports of Perry (1963) and Georgiev (1963) which show a reduced sensibility to Actinomycin action of an RNA with DNA-like base composition. The interference exerted on the incorporation of labeled precursors into RNA should be related to the inhibition exerted by Da on the activity of DNA-dependent RNA polymerase (Hartmann *et al.*, 1964; Ward *et al.*, 1965) (Fig. 17).

As observed for Actinomycin, the degree of enzyme inhibition increases with the antibiotic concentration and it can be comparatively antagonized by DNA.

How far could the inhibition of RNA synthesis be related to Da antimitotic activity?

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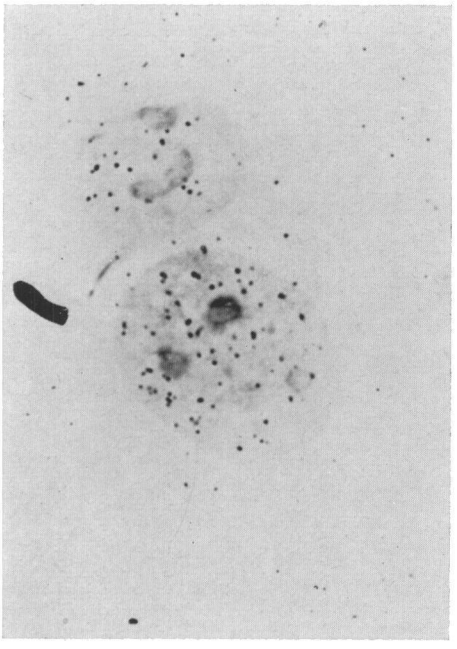

Fig. 12. Autoradiography of Hela cells after a 45' treatment with Actinomycin D 0,1 μg/ml and a 30' contact with ^{3}H uridine

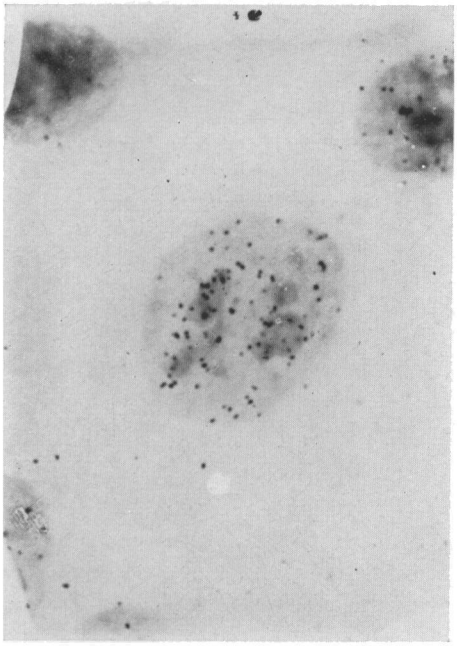

Fig. 11. Autoradiography of Hela cells after a 30' contact with ^{3}H uridine

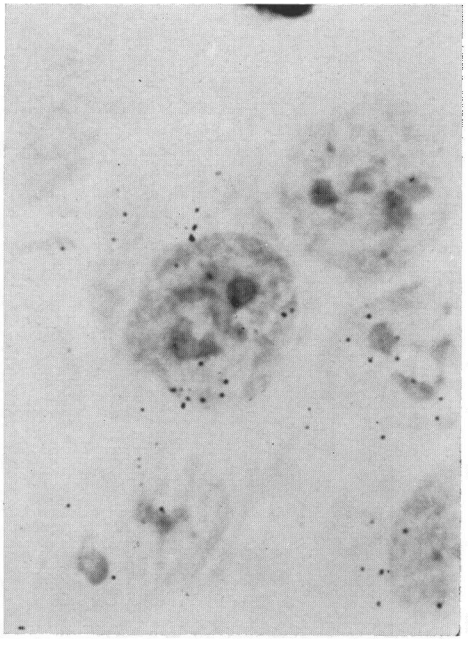

Fig. 13. Autoradiography of Hela cells after a 45' treatment with Actinomycin D 1 μg/ml and a 30' contact with ^{3}H uridine

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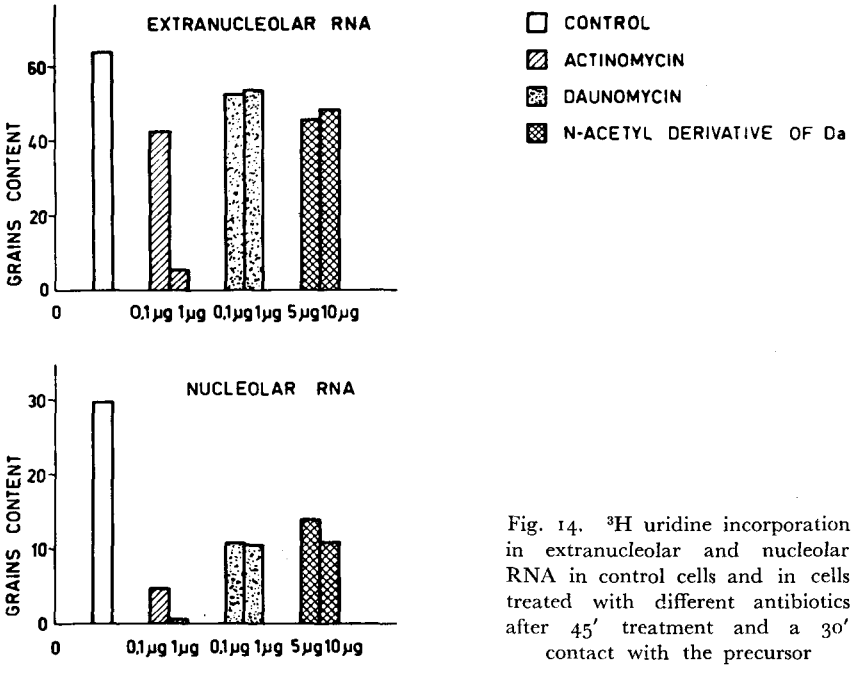


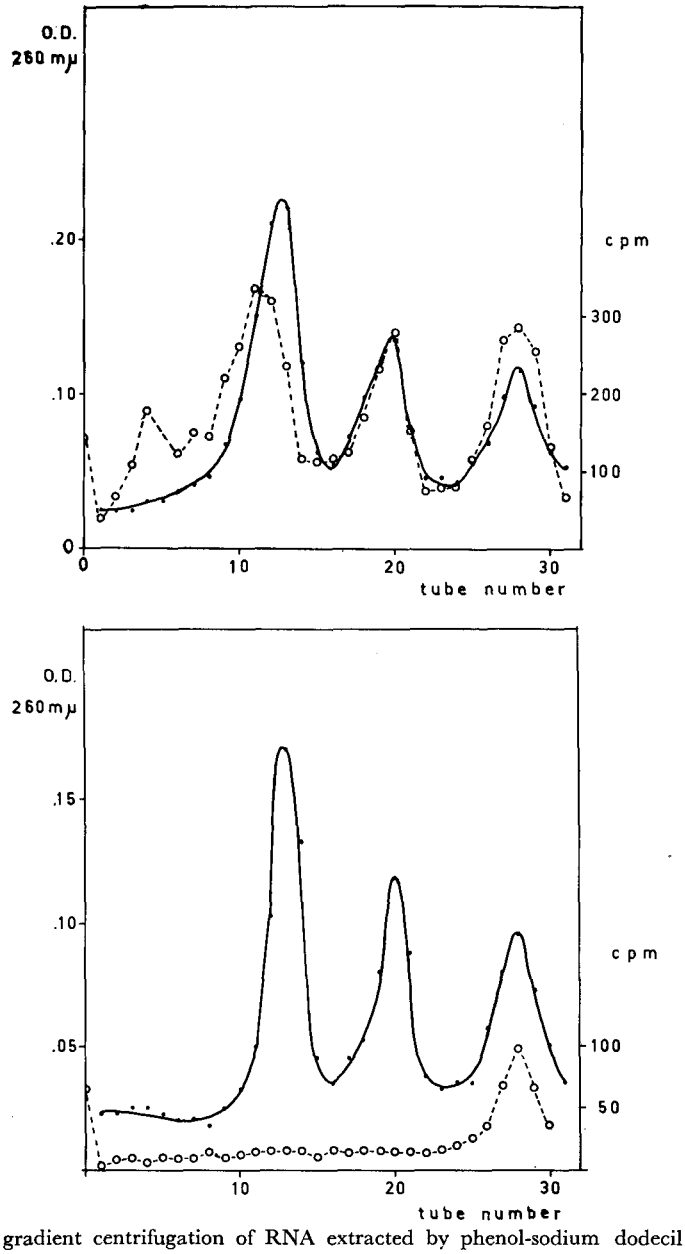
Fig. 14. ^{3}H uridine incorporation in extranucleolar and nucleolar RNA in control cells and in cells treated with different antibiotics after 45′ treatment and a 30′ contact with the precursor

Being the protein synthesis ultimately dependent on messanger and ribosomal RNA, it is evident that an inhibition of RNA synthesis would impair the reproductive capacity of the cell. But the sense of the posed question is whether this antibiotic inhibits the synthesis of RNA specifically related to DNA duplication and in general to cell reproduction. As such, one should consider messanger RNA responsible for the initiation of the synthesis of the enzyme requested for DNA duplication (thymidine kinase, DNA polymerase etc.) or for the synthesis and assembly of spindle proteins.

To answer the question (Di Marco *et al.*, unpubl.) we have devised the following experience: synchronized cultures of rat fibroblasts were treated with Da for one hour periods from the 9th to the 20th hour, labelled from the 21st to the 26th hour and fixed at the 27th hour.

It results (Fig. 18) that a reduction of the mitotic index is present in cultures treated from the 13th hour after the end of the mitotic wave on, therefore on cells which were near the middle of the interphase.

A reduction of both percentage of labelled cells and number of grains per nucleus is present in cells treated at the 9th hour (and that could be in the S phase) (Fig.19); thereafter the reduction of the values becomes less important, in fact the number of grains increases in the next two intervals. Only at the 15th hour the reduction in



Figs. 15-16. Sucrose gradient centrifugation of RNA extracted by phenol-sodium dodecil sulphate method; RNA was centrifuged on a preformed sucrose gradient; in the bottom of the tube 0.5 ml of 60% sucrose was placed in order to detect the RNA sedimenting at a rate higher 30 S. Incubation time: 120 min. The antibiotic was added 45 min before the labeled precursor

o-o-o O. D. 260 mμ; ^{0}- -^{0} counts/min. Fig. 15. Control Fig. 16. Daunomycin 1 μg/ml

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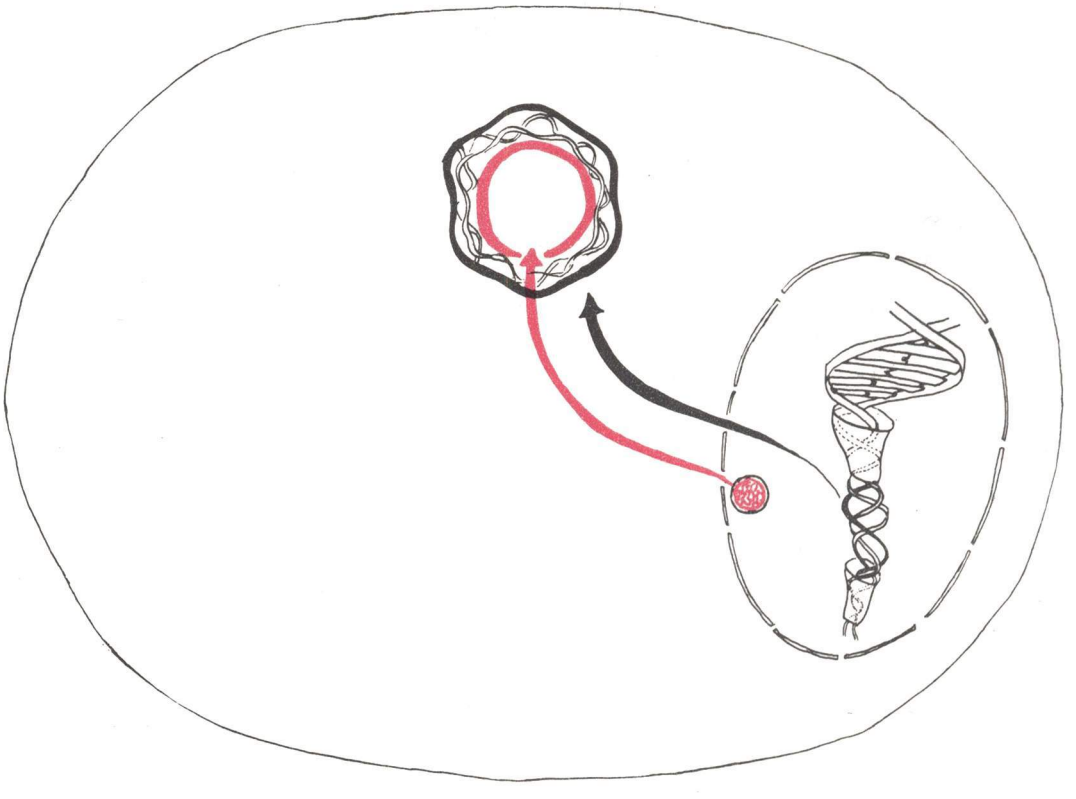

Fig. 17. Nucleolar and chromosomal origin of RNA present in ribosomes

labelling acquires new importance. These observations are consistent with the hypothesis that a series of biochemical events essential to DNA duplication begins in the cell near the middle of interphase. As these events can be influenced by an inhibition of DNA-dependent RNA synthesis, they could be equated with the formation of RNA molecules bearing the information wanted to synthesize proteins essential to cell division.

It is important to notice that the number of mitotic anomalies in this experiments is very limited in comparison with cells treated during S or G2 phases.

DNA synthesis

An interference of Da with DNA synthesis was also observed, following the incorporation of 8-^{14}C adenine into nucleic acids (extracted with NaCl according to procedure of Davidson and Smellie, 1952) on cell suspension of Yoshida's ascites

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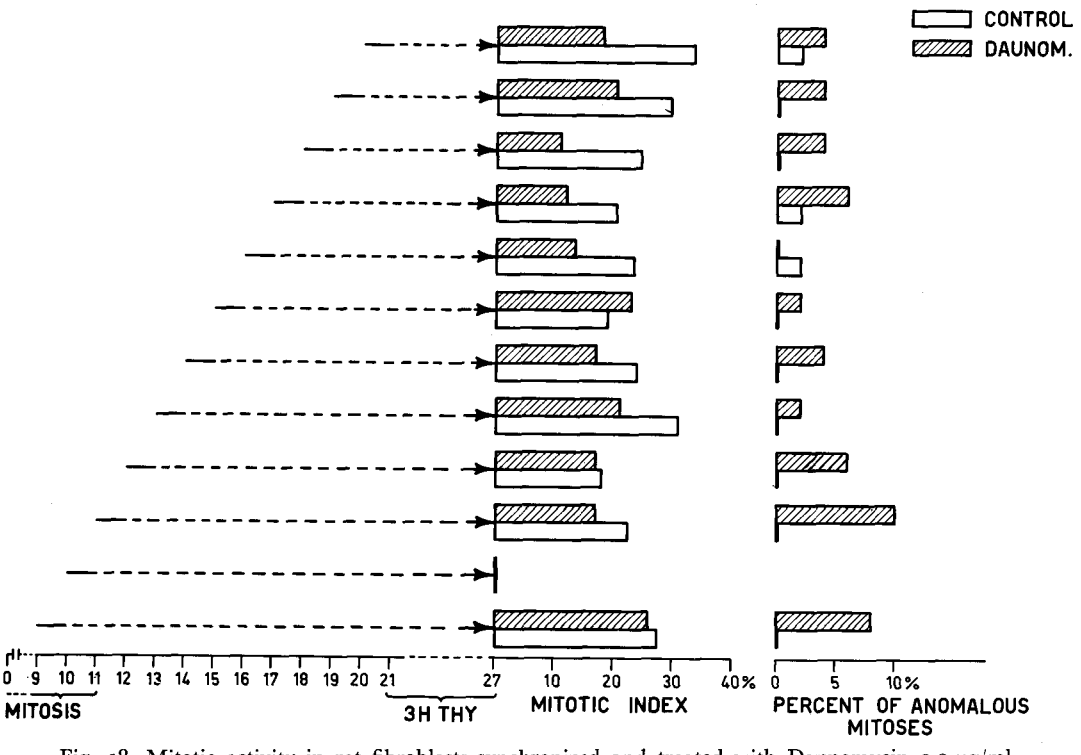

Fig. 18. Mitotic activity in rat fibroblasts synchronized and treated with Daunomycin 0.2 μg/ml at different intervals

hepatoma (Rusconi and Calendi, 1966) and on *in vitro* cultured Hela cells (Rusconi and Calendi, 1966) (Fig. 20).

By autoradiographic technique a marked interference of Daunomycin (Fig. 21) could be observed with the incorporation of ^{3}H thymidine into DNA of Hela cells cultured *in vitro* (Silvestrini *et al.*, 1963). DNA synthesis decreases proportionally to the dose used (0.1 μg/ml) but, in contrast to what has been observed in experiments carried out for comparative purposes with Actinomycin (Fig. 22) treated cells, it is not complete even with doses which rapidly stop mitotic activity (Di Marco *et al.*, 1965).

The inhibiting effect caused by Daunomycin on the incorporation of nucleic acid precursors into DNA should be related to the interference exerted by this substance on the activity of DNA-dependent DNA polymerase (Hartmann *et al.*, 1964).

The effect exerted by Daunomycin is strongly dependent on DNA concentrations in the medium. From the competitive effect between Da and DNA present in the inhibition of DNA synthesis we can presume that this inhibition also is to be ascribed to the formation of a complex between antibiotic and DNA (Fig. 23) (Calendi *et al.*, 1965).

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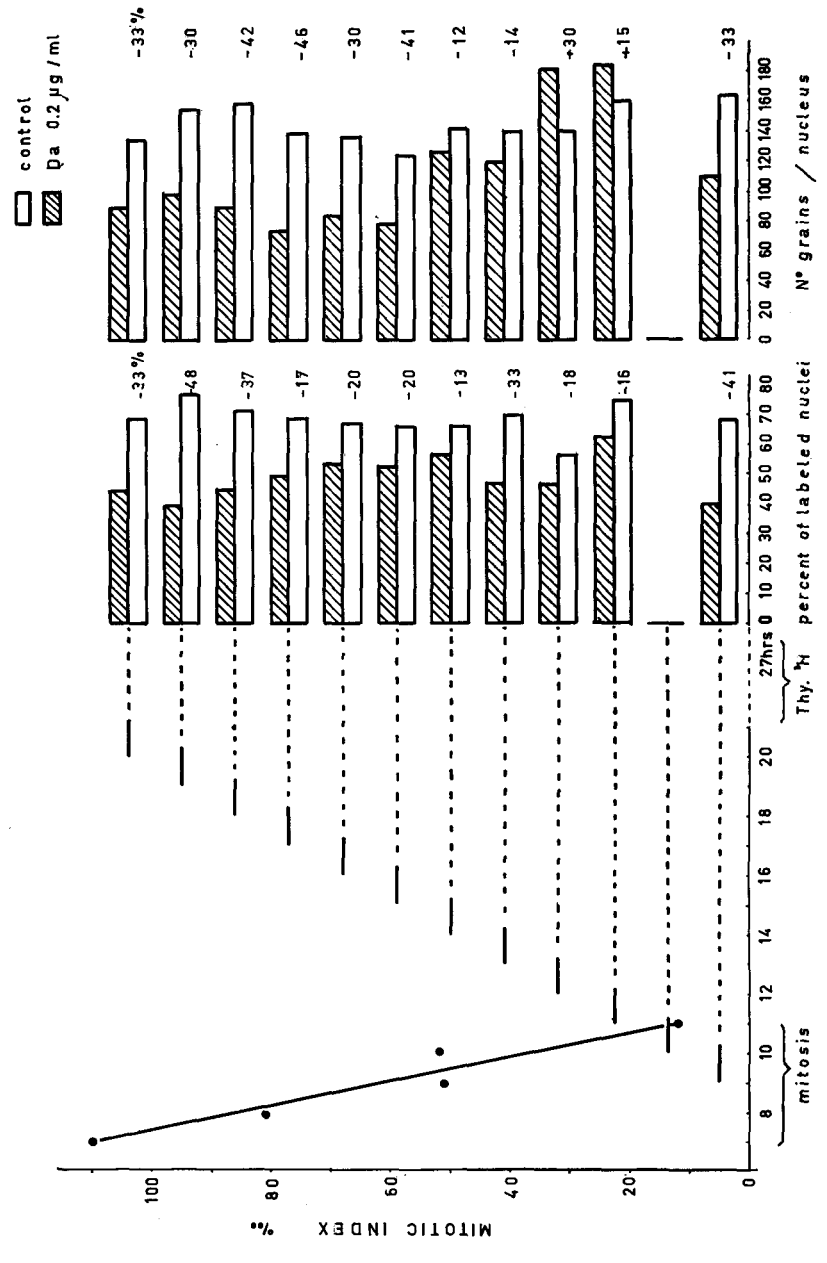

Fig. 19. Incorporation of ^{3}H thymidine in rat fibroblasts synchronized and treated with Daunomycin 0.2 µg/ml at different intervals

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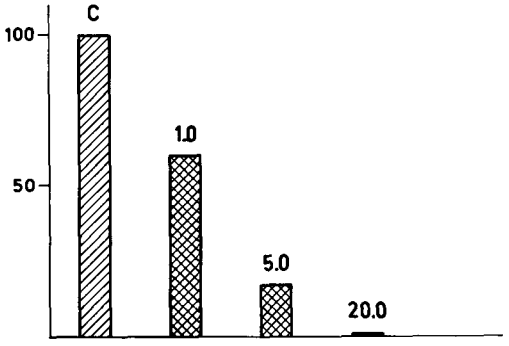

Fig. 20. Action of increasing doses of Daunomycin on the incorporation of Adenine 8-^{14}C into DNA of Hela cells

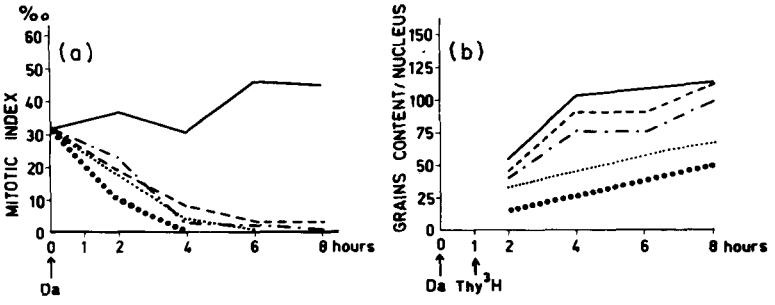

Fig. 21. *a*) Variation of mitotic index after treatment with different doses of Daunomycin
b) Thymidine 3H incorporation into control cells and Daunomycin treated cells
Control —; 0.1 μg/ml - - -; 0.2 μg/ml - · -; 0.5 μg/ml · · ·; 1 μg/ml ooo;

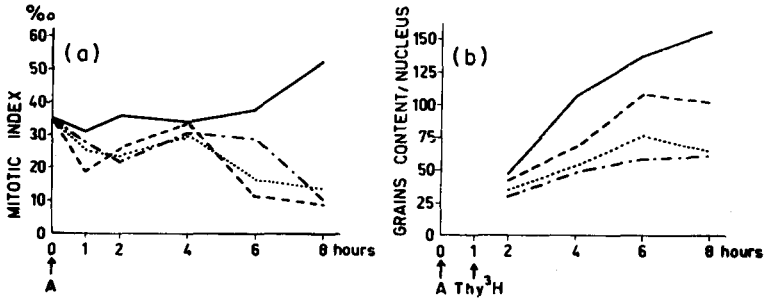

Fig. 22. *a*) Variation of mitotic index after treatment with different doses of Actinomycin D
b) Thymidine ^{3}H incorporation into control cells and Actinomycin D-treated cells
Control —; 0.1 μg/ml - - -; 0.5 μg/ml · · ·; 1 μg/ml ooo

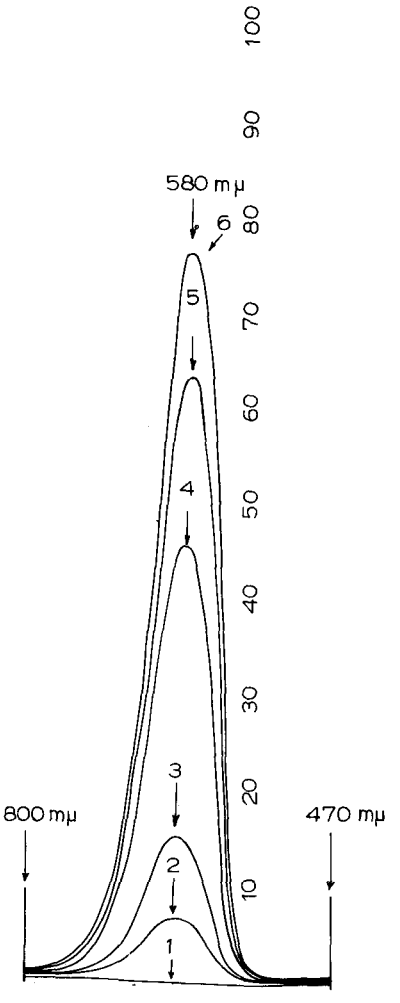

Fig. 23. Fluorescent emission spectra of Da in the presence of different levels of DNA

1) Blank; 2) 20 μg/ml + DNA 20 μg/ml 3) Da. 20 μg/ml + DNA 100 μg/ml
4) Da 20 μg/ml + DNA 50 μg/ml 5) Da 20 μg/ml + DNA 25 μg/ml 6) Da 20 μg/ml

The planarity of the anthracene-like structure present in the antracycline cromophore should have considerable influence on the complex formation with DNA favouring hydrophobic interaction of the ring system with theba se pairs. In strict analogy with the hypothesis of an intercalation of acridine dyes between adjacent base pairs (Lerman, 1964) an intercalation of Daunomycin between adjacent base pairs of DNA was suggested (Ward *et al.*, 1965).

Trying to understand the molecular mechanism of interference of anthracyclines with DNA replication two different possibilities should be considered:

a) the binding of the antibiotic to DNA causes a steric hindrance to the formation of the hypothetical DNA-DNA polymerase complex;

b) taking as granted that strand separation is a necessary requirement to the function of catalytic system (Bollum, 1963) the observed tendency of these substances to tie together the two strands of DNA molecule should inhibit the very beginning of the polymerization reaction.

Is the antimitotic activity of Da related to the reported inhibition of DNA synthesis?

Trying to answer the question we have made some experiences in synchronized cultures of rat fibroblast treated with Da following the program reported in Fig. 24.

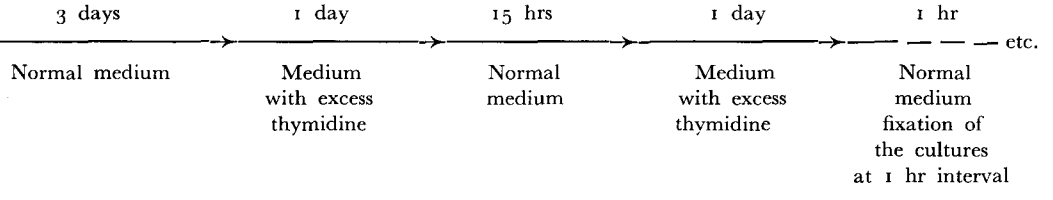

Fig. 24. Scheme of synchronization with double treatment with excess thymidine

In the first experiment the treatment covers a limited portion of the synthetic phase (Fig. 25) but owing to the tenacious binding of Da to DNA the antibiotic is probably present in the cells from the moment of addition on. The slide showed that the treatment of the cultures with a concentration of 0.2 μg/ml Da produces a marked drop in number of cells which enter mitosis (Figs. 26-27).

That the impairment in mitotic ability of these treated cells could be a consequence of the interference with the DNA synthesis is shown by the reduction in the proportion of labelled cells in the group which was treated and labelled at the same time (Fig. 28).

Some data, however, point to a possible dissociation between the antimitotic activity and the inhibiting effect on DNA replication.

In fact, in non synchronized cultures of Hela cells (and cells of tissue cultures M. C. sarcoma of rat) a remarkable inhibition of M. I. can also take place with Da doses slightly active on thymidine incorporation into DNA (Di Marco *et al.*, 1965).

In addition, it appears from the data reported in Fig. 26 that the maximum of efficiency in mitotic blocking is obtained when the treatment is postponed at the 5th to the 7th hour. This behaviour clearly indicates that the point of highest sensibility to Da lies in the phase G_2. (As previously mentioned, direct phase contrast observations showed in fact that Da can stop the mitotic activity of single cells when added until 20 minutes prior to the prophase stage). The biochemical knowledge of this part of the cycle is so poor that it would be improductive to make any hypothesis

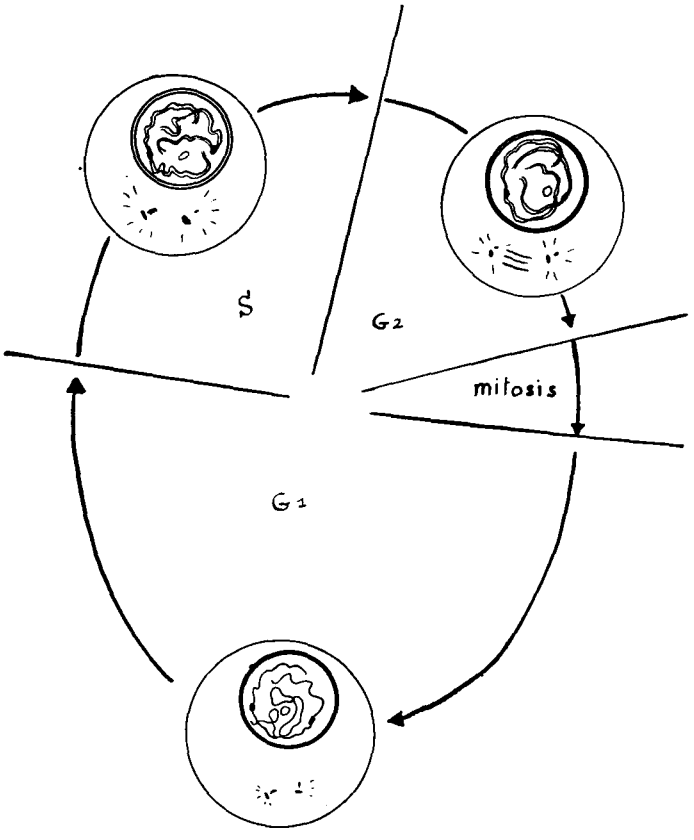

Fig. 25. Cellular regenerative cycle

on the nature of this peculiar interference. I would mention in this connection the observed antagonism of thio-compounds (such as thyoglycolic acid and cystein) towards the antimitotic activity of Da which could have some importance in view of the very well known role of sulfhydryl groups in mitotic process (Di Marco and Dasdia, 1966). Is the antimitotic effect also dependent on the ability of Da to form complexes with DNA?

It must be noticed in this connection that every modification of the structure of Da that impairs the binding capacity to DNA is also very effective on the antimitotic activity. This is considerably reduced in N-acetyl derivative of Da endowed with a low binding capacity to DNA (Tab. 1). Furthemore, thiol-compounds that antagonize the effect of Da on mitosis are also able to hinder the complex formation with DNA.

It is therefore possible that not only the chromosomal aberrations but also the

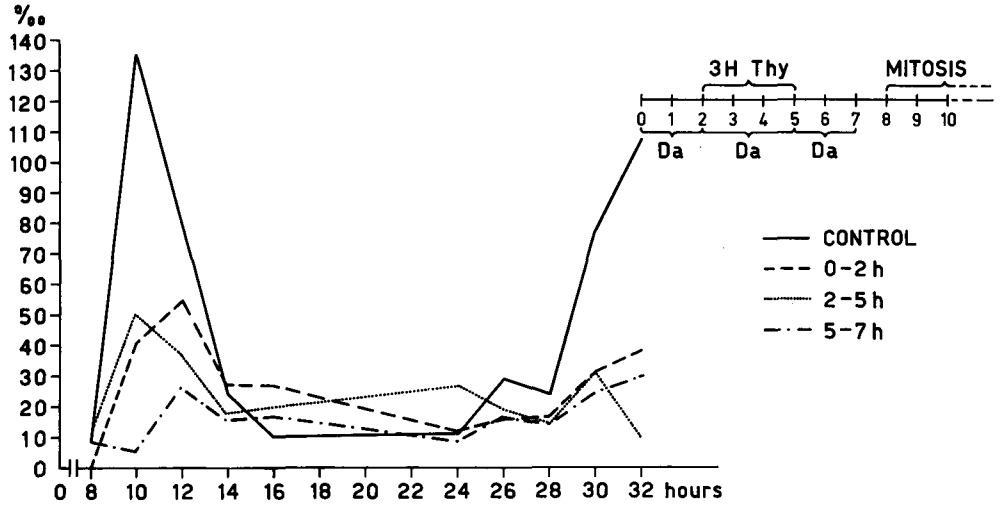

Fig. 26. Mitotic activity in rat fibroblasts synchronized and treated with Da 0.2 μg/ml at different intervals (0-2; 2-5; 5-7 hrs)

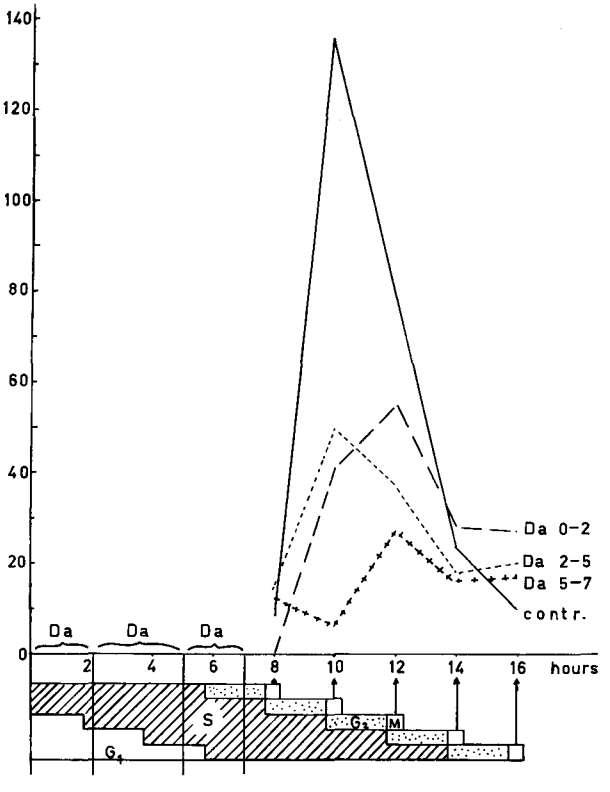

Fig. 27. Detail of Fig. 26

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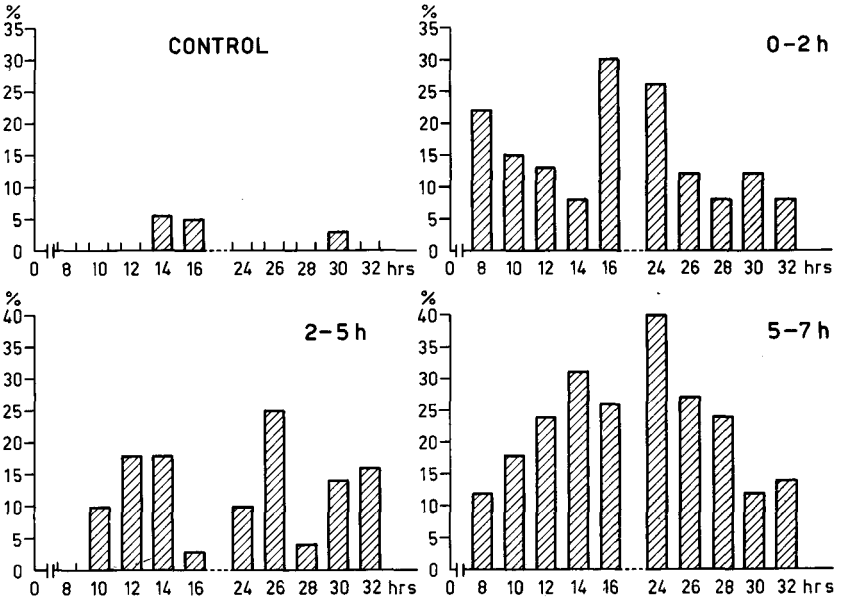

Fig. 28. Percent of labeled nuclei in cells synchronized and treated with Da 0.2 μg/ml at different intervals (0.2; 2-5; 5-7 hrs)

Tab. 1. Percent of inhibition in Hela cells treated with Da and N-acetyl-derivative of Da (Mitotic index; RNA and DNA synthesis)

	Mitotic Index			RNA Synthesis			DNA Synthesis		
	Dose	Time		Dose	Time		Dose	Time	
	μg/ml	2hrs	8hrs	μg/ml	45' + 30' nucleol.	extran.	μg/ml	2hrs	8hrs
Daunomycin	0.1	58.5	90.7	0.1	64	16	1	46	65.5
N-Acetyl Derivative of Daunomycin	40	60.2	69.8	5	59	16	40	0	0
	Da/N-acethyl der. 1 : 400			Da/N-acethyl der. 1 : 50					

impairment in chromosome movements be related to modifications of the chromosomal structure caused by Da.

Mitotic anomalies and chromosomal aberrations, such as three-group metaphase, laggard chromosomes, chromosome breaks, chromatide breaks with ring formation, true anaphasic bridges are frequent in cultures taken at different intervals from the treatment. These anomalies are also present in cells taken at the second mitotic

wave, and are probably expression of a permanent damage undergone by cells which escaped mitotic block. Evidence of the damage is also the reduction of labelling degree observed on the 32nd hour in cells labelled from the 2nd to the 5th hour and thereafter treated from the 5th to the 7th hour (Fig. 28).

In fact, the drop in number of grains per nucleus in this case means degradation by enzimatic activity of chromatinic material.

From these experiences it should be concluded that the binding of Da to DNA has manifold consequences depending on the physiological working of the cell at the moment of the contact with the drug.

Besides hindering the DNA's duplication, this impairs formation of RNA molecules which bear the information wanted to the building of new cells and interferes with the operation of the mitotic mechanism.

Summary

Daunomycin (Da) a metabolite of *Streptomyces peucetius* has a cytotoxic and antimitotic activity on normal and neoplastic mammalian cells grown *in vitro* and strongly inhibits a variety of experimental tumors.

The cell damage induced by Da is mainly nuclear.

A finely granular appearance of the chromatin and marked alterations in shape and size of nucleoli in resting cells and many mitotic aberrations in mitotic cells were observed.

Biochemical and autoradiographic researches show that the binding of Da with DNA causes a reduction of the DNA and RNA synthesis, especially of the RNA which is synthesized in the nucleoli and then passes into the cytoplasmic ribosomic RNA.

Treatment with Da on synchronized cells shows a marked drop of the mitotic index and appearance of mitotic anomalies when the cells are treated in S and G_2 phases.

This reduction keeps also in the second mitotic wave. A reduction of the mitotic activity is observed also if the cells are treated near the middle of the interphase.

Da causes a reduction in the percentage of the nuclei labelled with ^3H thymidine and in the number of grains per nucleus, when the cells are treated in S phase, in the very beginning and middle of interphase.

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RIASSUNTO

La Daunomicina, un metabolite dello *Streptomyces peucetius,* esercita un'attività antimitotica e citotossica su cellule normali e neoplastiche di mammifero coltivate *in vitro* ed inibisce fortemente vari tipi di tumori sperimentali.

Il danno che la Daunomicina provoca nelle cellule è prevalentemente nucleare.

La comparsa di cromatina finemente granulare e notevoli alterazioni nella forma e nelle dimensioni dei nucleoli è stata osservata nelle cellule in interfase come pure molte aberrazioni mitotiche in cellule in mitosi.

Ricerche biochimiche ed autoradiografiche mostrano che il legame che la Daunomicina contrae con il DNA provoca una riduzione nella sintesi di DNA e nella sintesi di RNA, specie di quello che viene sintetizzato nel nucleolo e passa poi al citoplasma come RNA ribosomico.

Il trattamento con la Daunomicina su cellule sincronizzate mostra una notevole caduta dell'indice mitotico ed una comparsa di anomalie mitotiche quando le cellule sono trattate nel periodo S e G_2.

Tale riduzione si conserva anche durante la seconda ondata mitotica.

Una riduzione dell'attività mitotica si osserva anche se le cellule sono trattate vicino alla parte centrale dell'interfase.

La Daunomicina provoca una riduzione nella percentuale dei nuclei marcati con timidina ^3H e nel numero dei grani per nucleo, quando le cellule sono trattate nel periodo S, nella fase iniziale e centrale dell'interfase.

RÉSUMÉ

La Daunomycine, un métabolite du Streptomyces peucetius, exerce une activité antimitotique et cytotoxique sur des cellules de mammifère normales et néoplasiques *in vitro,* et donc une inhibition remarquable de divers types de tumeurs expérimentales. Les dommages provoqués dans la cellule concernent surtout le nucléus,

La présence de chromatine finement granulaire et de remarquables altérations de la forme et des dimensions des nucléoles a été observée chez des cellules en interphase, ainsi que plusieurs altérations de la mitose. Les recherches biochimiques et autoradiographiques démontrent que la liaison de la Daunomycine avec le DNA est responsable d'une réduction de la synthèse de DNA et de RNA (surtout celui synthétisé dans le nucléole et qui passe ensuite dans le cytoplasme devenant RNA ribosomique).

Le traitement avec Daunomycine de cellules synchronisées démontre une baisse remarquable de l'index mitotique et la présence d'altérations de la mitose si le traitement a lieu dans les périodes S et G_2. Cette réduction se mantient aussi au cours de la deuxième vague mitotique. Une réduction de l'activité mitotique se produit aussi si les cellules sont traitées au milieu de l'interphase.

La Daunomycine cause aussi une réduction du pourcentage des noyaux marqués avec thymidine ^3H, ainsi que du nombre de grains par noyau, lorsque les cellules sont traitées dans la période S, au début et au milieu de l'interphase.

ZUSAMMENFASSUNG

Der Metabolit des *Streptomyces peucetius* namens Daunomycin übt auf normale und neoplastische *in vitro* gezüchtete Säugetierzellen antimitotische und zytotoxische Wirkung aus und inhibiert verschiedene Typen von Versuchstumoren stark.

Der Schaden, den Daunomycin in den Zellen bedingt, trifft hauptsächlich den Zellkern.

Bei den in der Interphase begriffenen Zellen beobachtete man das Auftreten feinkörnigen Chromatins und erhebliche Alterationen in Form und Dimension der Nukleolen; bei in Mitose befindlichen Zellen auch viele Mitose-Aberrationen.

Biochemische und autoröntgenographische Forschungen zeigen, dass die Bindung des Daunomycins mit der DNS eine Reduktion der DNS-Synthese bewirkt sowie eine Reduktion der RNS-Synthese, vor allem derjenigen, die im Nukleolus aufgebaut wird und dann als ribosome RNS ins Zytoplasma übergeht.

Bei Behandlung synchronisierter Zellen mit Daunomycin fällt der Mitoseindex erheblich und, wenn die Zellen in der S– und G_2–Periode behandelt werden, treten mitotische Anomalien auf.

Diese Reduktion bleibt auch während der zweiten Mitosewelle bestehen.

Eine Verminderung der Mitoseaktivität zeigt sich auch, wenn die Zellen in Nähe des zentralen Teils der Interphase behandelt werden.

Daunomycin vermindert den Prozentsatz der mit ^3H Thymidin gekennzeichneten Zellkerne und, wenn die Zellen in der S–Periode, in der beginnenden oder zentralen Interphase behandelt werden, die jeweilige Anzahl von Zellkörnchen im Kern.