Scientific Basis of Cancer Chemotherapy

Edited by
Georges Mathé

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Opening of the Inaugural Session by Mr. Maurice Schumann, Minister for Scientific Research

With 60 Figures

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Seminar on the Scientific Basis of Chemotherapy in the Treatment of Cancer in Man.
Organized by the Organisation Européenne de Recherche sur le Traitment du Cancer (OERTC).
Centre National de la Recherche Scientifique, Paris, 22—23 March, 1968

GEORGES MATHÉ, Professeur de Cancérologie Expérimentale à la Faculté de Médecine de Paris,
Directeur de l'Institut de Cancérologie et d'Immunogénétique, Chef du Service d'Hématologie
de l'Institut Gustave Roussy, F-94 Villejuif

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Introduction

The European Organization for Research into Cancer Treatment (OERTC), founded in 1962 initially under the name Groupe Européen de Chimiothérapie Anticancéreuse (GECA), is an extramural European Institute for collecting, co-ordinating and encouraging the work of scientists researching into cancer treatment at 16 European institutes: 3 in W. Germany, 2 in Italy, 2 in Belgium, 3 in the Netherlands, 2 in the United Kingdom, 1 in Switzerland and 3 in France.

The first President of OERTC was Professor GEORGES MATHÉ, now succeeded by Professor SILVIO GARATTINI of Milan. The organization has a *Board of 20 Directors*, responsible for co-ordinating the entire range of activities and guiding research and experimental studies on animals, also the first human trials of new therapeutic agents which have been fully tested on animals. The *Co-operating Groups* carry out complementary testing involving large numbers of patients.

Thus, OERTC is not a "learned society" but an agency for co-ordinating work.

OERTC's annual plenary meeting for 1968 was held in Paris on 22nd and 23rd March. It was given over to an intensive study of the scientific basis of chemotherapy in the treatment of cancer in man. These two working sessions brought together members of all groups, both directing and co-operating. The first day was in effect a teaching seminar, open to all practitioners wishing to use chemotherapy in cancer, and explaining the scientific basis of such treatment. This seminar constitutes the present monograph.

The session was opened by Mr. MAURICE SCHUMANN, Minister for Scientific Research, who stressed the government's interest in biomedical research, particularly cancer research. He thanked the members of OERTC for setting an example of European co-operation in a difficult field where the most up-to-date methods have to be reconciled with established ethical standards, i. e. therapeutic experiments. Let us emphasize, moreover, that the rules drawn up by OERTC for therapeutic experiments are a model for the now indispensable discipline of clinical pharmacology.

Opening Session, 9.30 a.m., 22nd March, 1968. Centre National de la Recherche Scientifique, Paris

Speech by Mr. Maurice Schumann, Minister for Scientific Research

When I received Professor MATHÉ's invitation to open this seminar which has brought you all to Paris today, I accepted with the greatest pleasure.

Your meeting gives me yet another opportunity to reaffirm my interest in three themes which I have very much at heart:

the priority role of biomedical research;
the urgency of keeping up the fight against cancer;
the place of European collaboration in research.

Among all the areas of research, there is none to which I attach greater importance than biomedical research. In its human and social implications, it is the noblest and most rewarding of scientific disciplines. The French government has given proof of the value it places on biomedical research in budget allocations which have increased sixfold since 1958; as yet, spectacular advance shows no sign of slowing down.

A very special effort has been devoted to cancer research, and this most feared and fearful scourge of our time has been challenged by attacking it with all the weapons scientific progress has made available to us. The battle, as you well know since you are engaged in it, has been a hard one. So far, there have been few spectacular triumphs but there has been a steady advance in the form of encouraging results, expressed in a rate of cure (or prolonged remission) of 40%. This is enough to justify our hopes and spur our determination to carry on with cancer research.

Nevertheless, such research requires a considerable deployment of human and material resources, often exceeding those available to a single institute, or even to a nation, such as France. We must therefore turn to international co-operation and what is more natural for us, as Europeans, than to start to practise it within the bounds of Europe? Is there any worthier object for European collaboration than research, above all when the target of this research is cancer?

I am well acquainted with your organization — Professor MATHÉ has often talked to me about it. It has been in existence for 6 years and is a model of European co-operation. It is not just another learned society among many; it is a working group whose members are distributed through 12 European institutes.

Your objective is precise and limited: applied research on the treatment of cancer, whether by radio-therapy, immunotherapy or chemotherapy.

Up to the present you have devoted the greater part of your efforts to chemo-therapy. There seems no end to the ability of chemists to produce, either by synthesis or by extraction from natural materials, substances possessing pharmacodynamic properties, in your case active against cancer. The screening of these innumerable products creates immense problems which require the resources of a continent to solve them. Thanks to your co-operating groups, you are able to carry out therapeutic trials on patients in all parts of Europe, and it is one of the achievements of your

organization to have created a bond of solidarity, not only between doctors, but also between patients all over Europe.

The unique feature of your organization is that it grew out of a spontaneous action of scientists and doctors. You have to some extent anticipated government initiatives, guided only by the best interests of your patients and the demands of your discipline.

I am happy to pay tribute to your success!

Contents

Anti-Cancer Agents. Their Detection by Screening Tests and their Mechanism of Action

T. A. CONNORS [1]

With 10 Figures

The majority of the compounds, that the clinician uses today in the treatment of cancer, have been discovered because they showed activity in experimental screening systems.

One has a large number of experimental models to choose from when screening derivatives for anti-tumour effect [1]. They may, for instance, be tested on tumour bearing animals, against one of a whole host of microbial systems or cell cultures, or one may even use simple biochemical estimations as an indicator of anti-tumour activity (Table 1). The fact that a large number of screening systems are at our

Table 1. *Screening tests*

Spontaneous tumours	Laboratory animals
Induced tumours (viruses, chemicals)	
Transplanted tumours (ascites, solid leukaemias)	
Heterotransplanted human tumours	Hamster cheek pouch Conditioned animals Chorioallantoic membrane
Human tumours	Cell or organ Culture
Microbial systems	(e. g.) *L. casei, T. gelleii* (e. g.) Glycolysis inhibitors
Mutagenic compounds	
Inhibitors of immune responses	
Effects on peripheral blood	

disposal does not mean however that we can predict with confidence that a drug will be an effective anti-tumour agent in the clinic. Rather, the wide variety of tests used at present is an indication of the failure of any one test to prove completely reliable in selecting effective anti-tumour agents.

[1] Chester Beatty Research Institute, Royal Cancer Hospital Fulham Road, London, S. W. 3.

Spontaneous Tumours

The obvious choice of a test system for screening anti-tumour agents would appear to be one which uses laboratory animals bearing spontaneous tumours. Many strains of rat and mice are known which have a high incidence of a particular type of so

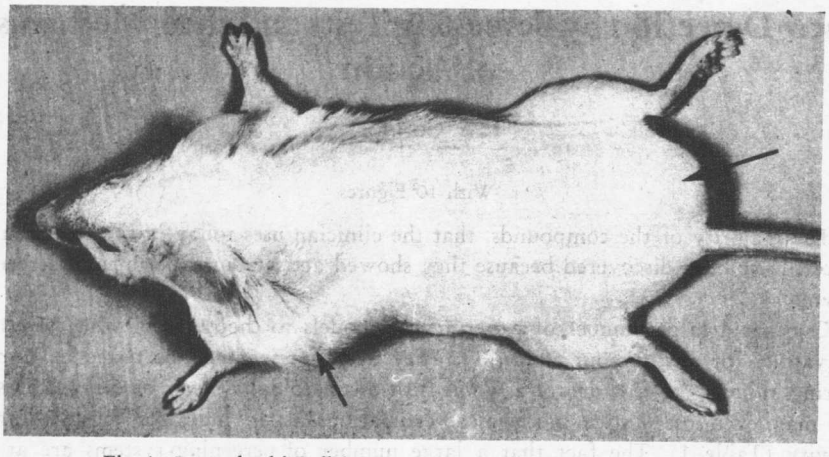

Fig. 1. 6 month old Balb/c$^+$ mouse with two mammary tumours

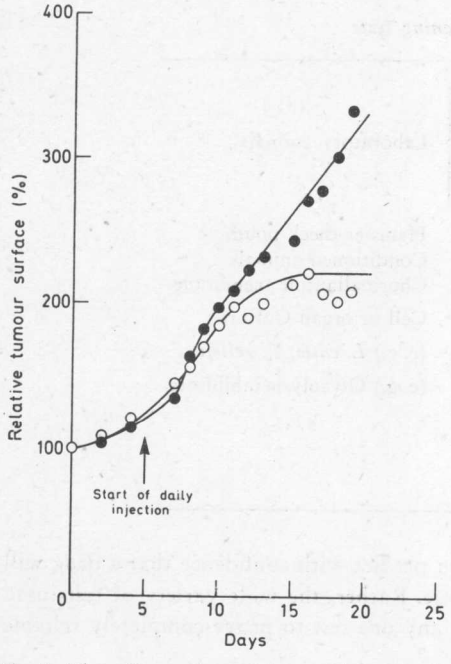

Fig. 2. The effect of xanthine oxidase on the growth of a "spontaneous" mammary tumour [After HADDOW, DE LAMIRANDE, BERGEL, BRAY and GILBERT (1958)]. ●—● Control; o—o treated

called spontaneous tumour, although of course, a number of these are now known to be viral in origin. Mammary tumours are particularly favourable for use as they can be readily detected at an early stage of their development when they are most likely to be sensitive to chemotherapy. Their growth rate can also be easily measured and the effect of agents in restricting their growth assessed. Fig. 1 shows Balb/C$^+$ mouse bearing two mammary tumours. Using these animals in a screening test one would, in the simplest system, compare the growth rate of the tumours of a control group of animals with the growth rate of a group animals treated with the drug under test. In practice, since the tumours arise in different sites and there may be more than one per animal (as shown in Fig. 1), the tumours of treated and control groups are matched before the test begins. Fig. 2 shows the result of a test where the anti-tumour effect of the enzyme xan-

thine oxidase was assessed [2]. The growth rate of the tumours of two paired groups of mice were measured, and 6 days after the measurements had begun, one group received daily injections of the enzyme, while the other group received solvent only. The compound is classed as active since it held up the growth of the tumours during the course of treatment. Growth of the tumour may be expressed either as surface area or volume and these values are obtained from caliper measurements of the tumour in at least two directions followed by the appropriate calculations [3]. Tests such as these using mammary tumours have been described by a number of authors [4, 5, 6].

Induced Tumours

A more convenient system to handle is one which uses chemically induced tumours. In such a system, the tumours can be made to arise in more accessible areas. They are often encapsulated and spherical in shape and these factors all enable the tumour volume or surface area to be calculated with less error than with the spontaneous tumours described above. Fig. 3 shows a white rat with a fibrosarcoma induced in

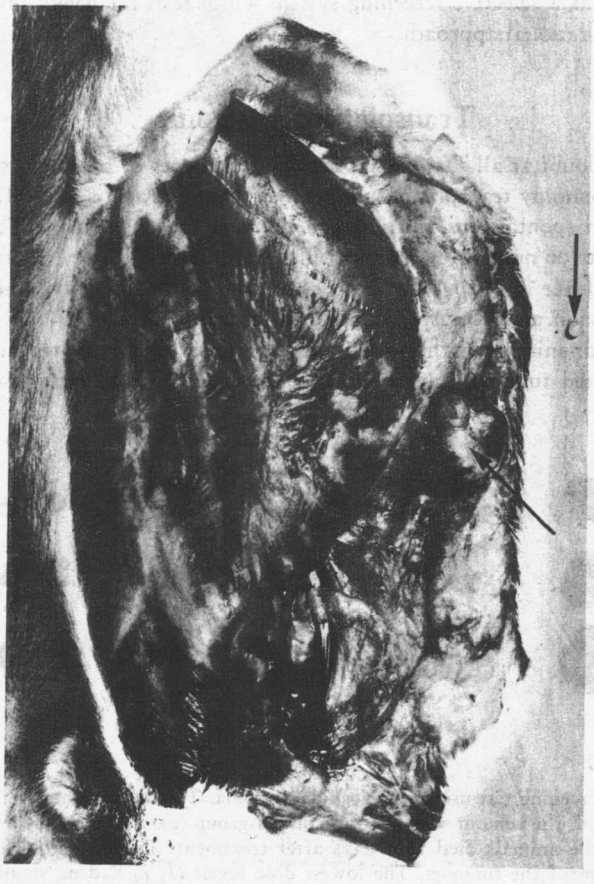

Fig. 3. Fibrosarcoma induced by a pellet of benzpyrene after a latent period of 6 months

the flank by a pellet of benzpyrene. Basically, compounds are tested for their anti-tumour effect against induced tumours in the same way as that already described for spontaneous tumours [7, 8, 9, 10]. A variation of the technique is to assess the effect of drugs in delaying the appearance of tumours in animals treated with a carcinogen [11]. Where the tumour induced is a leukaemia, the effect of a drug may be measured by its ability to reduce the differential white cell count and prolong survival time [12].

In practice one rarely sees either spontaneous or induced tumours used on a large scale as screening tests. The main reason for this is simply a practical one. Induced or spontaneous tumours usually arise at the earliest when the animals are between six and nine months old. Even if the specialised approach of injecting a carcinogen neonatally is employed the latent period before the tumour occurs is of the order of 3 months. Not all the tumours develop at the same time and, even if the tumour incidence is high, it means that, using the modest quantity of 100 animals a month for screening, space for at least 1000 animals with developing tumours must be provided. One usually finds that these kinds of screening system are employed either as secondary screens, that is to test further compounds already found to be active by other means, or as a selective screening system which tests relatively few compounds selected by some rational approach.

Transplanted Tumours

There is no doubt at all that the most important types of screening test at present are those which employ transplanted tumours growing in rats or mice. The difficulties experienced with spontaneous and induced tumours no longer arise. The size of the tumour fragment (or number of tumour cells) transplanted may be controlled so that the tumour incidence is 100%, the tumours are similar in size, have uniform growth rates and arise soon after transplantation. No animals are wasted and little space is required to hold animals with developing tumours. Fig. 4 shows an experiment where transplanted tumours have been used to screen a drug. Forty-two white rats

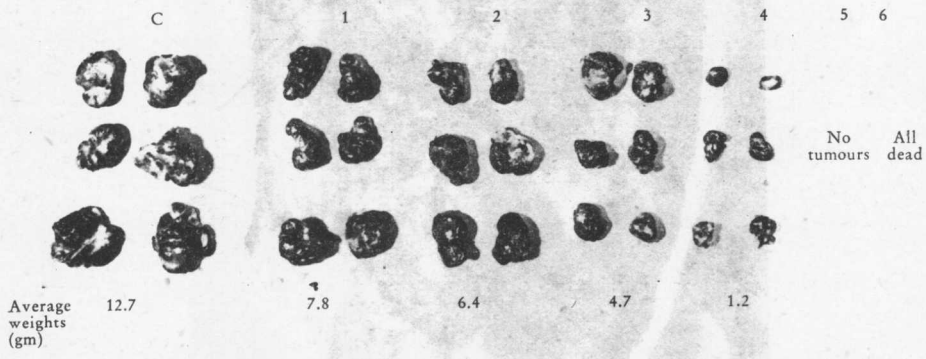

Fig. 4. Result of screening test using the Yoshida sarcoma. Tumour weights of treated animals were compared with the tumour weights of a control group (c). After the highest dose level of the drug (6) all the animals died four days after treatment. The next dose level (5) caused complete regression of the tumours. The lowest dose levels (1, 2) had no significant effect on the growth of the tumour

were transplanted subcutaneously with two million Yoshida sarcoma cells. One week later, the animals had solid tumours weighing between 1.5 and 2.5 gms [13]. At this time they were randomly divided into seven groups. One group served as untreated controls and the other groups received the drug under test at various dose levels. One week after this treatment, when the tumours were now 14 days old, the animals were killed and the tumours dissected out and weighed. Fig. 4 shows the tumours at this time: one can see that the highest dose level of the drug used was lethal killing all six animals. The next dose level, the maximum tolerated dose, caused complete regression of the tumour while the next two doses had some effect on tumour growth. The two lowest doses had little or no significant effect on the growth of the tumour.

Screening tests such as these must answer one of two questions. Firstly, in the case of a completely new class of compound, does it inhibit the growth of the tumour at its maximum tolerated dose? Secondly, in the case where the compound tested is an analogue of derivatives previously shown to have activity against the tumour, one must ask not only is the compound a tumour inhibitor but also how well does it compare with the previously tested members of the series? A quantitative comparison is best made by estimating the therapeutic index for each derivative. This index gives an indication of the selectivity of anti-tumour action of a compound and one can readily be calculated from the data given by the Yoshida sarcoma test as already described. Provided the highest dose used killed all the animals an LD_{50} can be calculated and provided there are some dose levels of the drug which cause little or no tumour inhibition, a dose to give 90% tumour inhibition (ID_{90}) can also be calculated. The ratio LD_{50}/ID_{90} is one form of therapeutic index and very roughly gives the degree of separation between the dose required to kill the majority of tumour cells and the dose required to kill the animal. A large number of different kinds of transplanted tumours are used or have been used as screening tests [1] and, generally, a similar testing procedure is employed to that described here. The antitumour effect is not necessarily measured by comparing tumour growth rates or tumour weights a certain time after treatment. One can estimate a dose required to eradicate the tumour completely, or by direct cell counting measure the number of cells killed by the drug. Survival time, or in the case of leukaemia, the effect on leukocyte count can be used as parameters of drug effectiveness. Certain ascites tumours have proved particularly valuable in more precise experiments where the survival time of treated animals can be directly related to the number of cells killed [14]. Table 2 demonstrates the screening procedure for the Walker carcinoma 256, one of the tumours used in the primary screen of the Chester Beatty Research Institute [15].

From a preliminary toxicity test the upper dose of 32 mg/kg has been selected knowing it will prove lethal to all the animals and enable the calculation of an LD_{50}. The day after transplantation of the tumour, six groups of rats have received a single injection of the drug under test at dose levels ranging from 32 mg/kg to 1 mg/kg. Ten days after injection of the drug, the tumours are weighed. The compound under test, a nitrogen mustard, has a therapeutic index of three, and since the most active nitrogen mustards have indices of 10—20 against this tumour, this particular derivative would probably not be considered for further screening tests. In order to obtain a more precise form of maximum tolerated dose, the body weight change of the animals is also recorded in this test.

Table 2. *Quantitative Walker tumour inhibition and toxicity test*

CB 1939 Name: O(NN-bis(2-chloroethyl)amino)phenol HCl Solvent: Arachis oil No. of injections: 1 Route of administration: i.p.
Date of implant: 6. 4. 67 Date of 1st injection: 7. 4. 67 Date killed: 17. 4. 67 Average weight of rats at commencement of test: 252 gms

Control untreated	Dose 1 mg/kg		Dose 2 mg/kg		Dose 4 mg/kg		Dose 8 mg/kg		Dose 16 mg/kg		Dose 32 mg/kg	
Tumour weights	Tumour weight	Body weight change	Tumour weight	Body weight change	Tumour weight	Body weight change	Tumour weight	Body weight change	Tumour weight	Body weight change	Tumour weight	Body weight change
gms	gms	gms	gms	gms	gms	gms	gms	gms	gms	gms	gms	gms
66	52	+42	53	+13	56	+12	7	+92	0	+44	Died	Day 4
59	43	+ 4	49	+48	29	+97	5	+70	0	−30	Died	Day 4
55	31	+11	34	+78	17	+84	0	+71	0	−24	Died	Day 5
53												
44												
Average body weight change												
+40 gms	C/T=1.3		C/T=1.1		C/T=1.3		C/T=13		C/T=∞		C/T=	

Screening tests carried out on transplanted tumours have in fact been responsible for the discovery of the majority of the chemicals used in the clinic today. Before 1946, it was not considered feasible to treat cancer by chemotherapy and, before large scale screening tests commenced around 1950, there were only a few compounds available which were occasionally effective in the treatment of some cancers. Since that time, as a result of screening against many types of animal tumour (some of the most notable being the L 1210 leukaemia, the sarcoma 180 and the Ehrlich ascites of the mouse and the Walker carcinoma and Yoshida sarcoma of the rat), we now have a large variety of different agents effective against particular kinds of human cancer. The effect ranges from the spectacular, such as the treatment of choriocarcinoma where 70% of patients are curable by chemotherapy alone, to the very good responses obtained in acute leukaemia in children (90% complete haematological remission with prolongation of survival time) and Burkitt's lymphoma (15% long term complete remission) and to the moderate responses of Hodgkin's disease reticulum cell sarcoma, myeloma and the chronic leukaemias (50—90% remissions) [16].

However, cancers of the lung and many cancers of the gastrointestinal tract are quite refractory to chemotherapy using the agents we have at present. Since cancer of these two sites are the most frequent cancers encountered in many countries, it has often been felt that transplanted tumours are proving inadequate in selecting chemical agents useful for the treatment of all types of cancer. Mainly for this reason, other screening models have been designed with the hope of discovering new classes of anti-tumour agent.

Other Kinds of Screening Test

In order, perhaps, to get closer to the human situation, a number of tests have been devised where the activity of the drug is assessed against human tumours. Human tumour cells may be transplanted into the hamster cheek pouch or into rats and mice with immunological defences first suspended by X-irradiation and then maintained in the depressed state by cortisone conditioning. There is no evidence at the moment that these interesting although technically tedious methods will produce results any more useful than the simple transplantation techniques already available [17, 18, 19].

Two different types of test using human cells in culture have also been described. In the first, human cell lines are used to screen drugs of unknown anti-tumour potential. Screening tests employing stock lines of human cells such as HeLa, J-111 and HEP 3 have been reported [20, 21]. However, there is no evidence that the use of human cells, which have been in culture for many generations, offers any advantage over screening tests using animal cells, many lines of which have been used to detect potential anti-tumour agents [22]. Of potential use is the second type of test using human cells in culture. In this test, human material taken at operation or by biopsy is submitted to the action of a range of known anti-tumour agents. The compound proving most toxic to the tumour tissue is then used for the treatment of the patient from whom the specimen was taken [23, 24]. In cases where this rather lengthy individual screening test can be carried out, it would seem to be a very rational approach for treatment. However, as will be mentioned in a later talk, the host can affect an administered drug in a variety of ways and this type of testing

may be particularly useful only when the drug is given by intra-arterial injection or regional perfusion [25]. By these techniques, the drug is brought into direct contact with the tumour cells similarly to the *in vitro* test.

Similar to tests using cell culture, the effect of drugs in direct contact with bacteria, bacteriophages, fungi, viruses and protozoa have all been advocated as tests for anti-tumour agents. However, there is no evidence that any of these systems could replace an *in vivo* tumour system as a screening test for anti-cancer agents [26], and there is every reason to believe that they suffer from disadvantages not found with screening tests using tumour bearing animals. As will be seen later, most of the known anti-tumour agents act by interfering with some stage of nucleic acid or protein synthesis and, since these processes are just as essential to cells in culture or micro-organisms, then such *in vitro* screens will show a certain correlation with the *in vivo* tests in the drugs they select. However, drugs like endoxan, one of the most useful of the alkylating agents, would be missed by such screens on account of its low toxicity *in vitro*. Many false positives might also be picked up by these screens since they do not measure the corresponding toxicity of the drug to control normal cells. The only justification for the use of these tests for primary screening of drugs for anti-cancer activity is where (a) the facilities are not available for tests using tumour bearing animals or (b) where such a large number of compounds are waiting for test that they cannot all be tested *in vivo*. It is, however, not a satisfactory situation where one has to rely on *in vitro* screening tests for the selection of compounds entering a screen using tumour bearing animals. At the best, the two tests should be run side by side since they can give complementary information on the anti-tumour action of drugs.

Correlations between enzyme activity and tumour growth rate, and degree of tumour inhibition by drugs and enzyme levels, has led to the formation of simple biochemical tests where the effect of drugs on particular enzyme systems is studied. In view of the various correlations observed, it is argued that drugs affecting the enzymes most will be the most effective anti-tumour agents. Tests have been designed for instance which measure the effect of drugs as inhibitors of glycolysis, lactic dehydrogenase and xanthine oxidase [27, 28, 29]. But none of these empirical tests could ever be expected to replace existing screening tests.

Classes of Anti-Tumour Agents

The classes of chemicals useful in the clinic are shown in Table 3, together with the best known examples of the class and the tumours which are known to respond best to treatment. However, despite the fact that widely differing chemical structures are represented, all of them with the possible exception of the hormones act by interfering at some stage of nucleic acid (mainly DNA) or protein synthesis. Their mechanisms of action are summarised in Fig. 5. In the biosynthesis of nucleic acids nucleotides are first formed from precursors present in the cell. These nucleotides are then polymerised in the appropriate sequence to form nucleic acids. Using DNA as a template, messenger RNA is formed and this together with the various transfer RNA's is responsible for the formation of proteins at the ribosomes. Some of the enzymes synthesised in this way are responsible for the synthesis of DNA and nucleotides. Anti-folics such as methotrexate prevent the formation of certain

nucleotides. Some purine and pyrimidine anti-metabolites also act in this way, others can prevent the incorporation of nucleotides into nucleic acid or may themselves be incorporated forming an abnormal nucleic acid molecule. Alkylating agents and mitomycin C undergo a direct reaction with DNA and prevent its continued synthesis. Actinomycin D is also associated with DNA but in this case it prevents the formation of messenger RNA with considerable effects on protein synthesis. This inhibition of protein synthesis will also eventually lead to a deficiency of the enzymes

Table 3

Class of compound	Examples	Tumours against which drug most effective
Anti-folic	Methotrexate	Acute leukaemia Choriocarcinoma
Anti-purines+Pyrimidines	6-Mercaptopurine 5-Fluorouracil	Acute leukaemia Breast carcinoma
Alkylating agents	Melphalan, Endoxan E 39, Thio-tepa Myleran	Multiple myeloma Lymphomas and leukaemias
Hormones	Oestrogens Androgens	Hormone dependent carcinomas
Steroids	Corticosteroids	Leukaemias and lymphomas Breast carcinoma
Anti-biotics	Actinomycin Rubidomycin Mitomycin	Wilms tumour Leukaemias and lymphomas
Plant extracts	Vincaleukoblastine Vincristine Colchicine	Hodgkin's disease Reticulum cell sarcoma

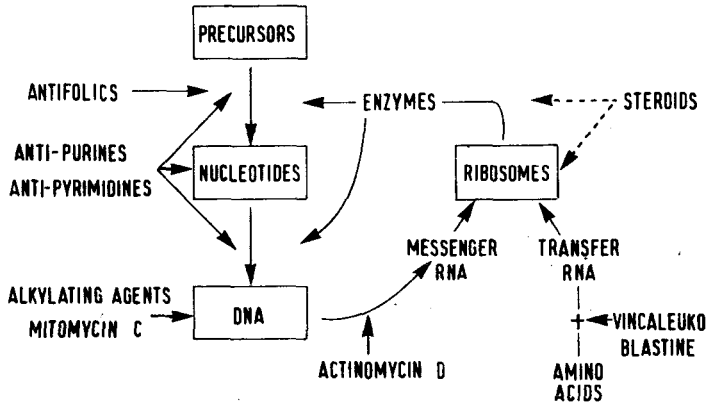

Fig. 5. The sites of action of some cancer chemotherapeutic agents

required for nucleic acid synthesis. Vincaleukoblastine also affects protein synthesis but in this case it appears to interfere with the function of certain transfer RNA's. The steroids have a rather obscure action on proteins and ribosomal RNA.

The Alkylating Agents

Many types of chemical alkylating agent are known but members of the nitrogen mustard, ethylenimine, epoxide and sulphonoxyalkane series are the only ones to have been shown to be anti-tumour agents. Fig. 6 shows the basic structures of these alkylating agents. An alkylation can be considered to be the replacement of the hydrogen atom of a molecule by an alkyl ($R.CH_2–$) group. The radical R may be a complex one (e. g. aromatic or containing functional groups) but the attachment to the molecule (HR′) must be made through a fully saturated carbon atom (i. e. $–CH_2–$) as shown in Fig. 6. Although the alkylating agents may react by more than one mechanism [30], for our purposes we can consider all of them to react by the formation of a reactive intermediate. The structures of the various alkylating agents differ widely, yet they all react by the intermediate formation of a positively charged carbonium ion ($–CH_2^+$), as shown in Fig. 6.

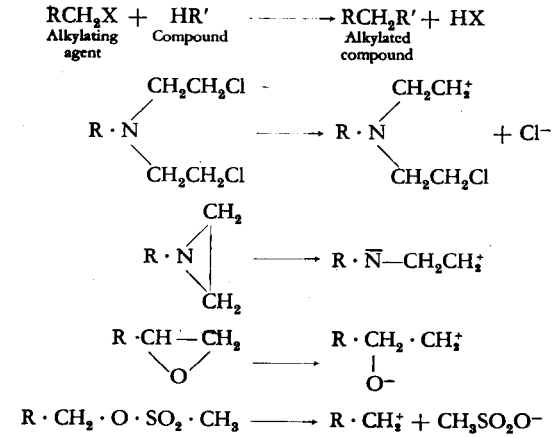

Fig. 6. The basic structures of some important antitumour alkylating agents. All these agents react after the initial formation of a positively charged carbonium ion ($-CH_2^+$)

This positively charged carbonium ion will then be highly reactive towards negatively charged centres such as ionised carboxylic and phosphoric acids, ionised thiol and hydroxyl groups and uncharged amines (Fig. 7). Groups such as these occur in many biologically important molecules, nucleic acids, enzymes, structural proteins, lipids and amino acids. It is obvious then that the alkylating agents have the possibility of reacting with very many different molecules inside the cells and they have, in fact, been shown to inhibit many pathways by alkylation of different compounds. However, by studying the effects of the alkylating agents at dose levels just sufficient to cause cell death, evidence has accumulated that DNA is the most sensitive molecule to alkylation. Many studies in model systems and whole animals indicate that alkylating agents kill tumour cells (and sensitive normal cells) by cross