CHAPTER 40

Antitumour Agents

T. A. Connors and F. J. C. Roe

A. Introductory

Current practice in testing potential cancer chemotherapeutic substances is in reality an unstable and somewhat unhappy compromise between the desirable and the practicable. Transplantable animal tumours form the keystone of most programmes. Everyone concerned

realises that this type of test system is several steps removed from the real problem of finding drugs effective against spontaneous cancer in man. The situation is not unique to testing for antitumour effect; it finds its counterpart in, for example, testing for psychopharmacological activity. Nevertheless, the practical difficulties of testing large numbers of substances by any method closer to the clinical problem seem at present insuperable. On first principles one would imagine, since it is undesirable or impossible to test new substances on man, that tests should be made on animals with spontaneous cancer. Long before the search for cancer chemotherapeutic agents became a major enterprise, cancer research workers selected the mouse and the rat, because of their short life span, as the most suitable species for large-scale experimentation. Consequently, from the start, many genetically homogeneous lines of animals, developed by brother-sister mating during more than 20 generations, were available for this type of study. Mice of some inbred strains were known to be particularly liable to develop distinct forms of cancer, in particular, leukaemia, mammary cancer, pulmonary adenomas, and hepatomas. Of these, leukaemia and mammary cancer have been used for testing drugs for antitumour activity (v. below, Sections E and F). In practice, however, such spontaneous tumours seem to have no advantages over transplantable ones, and possibly some disadvantages (V. R. Potter, 1961); the fact that viral agents are responsible for both these types of tumours (Friend, 1957; Bittner, 1936) may indicate that it would be unwise to regard them as models for picking out drugs effective in the treatment of human cancer. Perhaps, when it is known which forms of human cancer have a viral aetiology, it will be possible to use such tumours on a more rational basis. At present, virus-induced tumours are still being used, but never as the sole test system (v. below). There are indeed many "spontaneous" tumours for which a viral aetiology has not been demonstrated, but their use for test purposes is restricted, not only because of their unpredictable time of appearance and low incidence, but also because of the difficulty of diagnosing and measuring spontaneous tumours unless they happen to arise in accessible sites. Clearly it would be extremely costly to keep large numbers of animals until the latter part of their life-span in the expectation that perhaps only 20% would develop cancers, of which less than half would be in accessible sites.

It has therefore been necessary to take a further step towards artificiality, away from the clinical problem in the search for satisfactory test methods. It is possible to induce cancer regularly at various sites in animals by exposing them to physical, chemical or viral agents. Often the resulting tumours appear indistinguishable from "spontaneous" cancers at the same sites. In this connection the chemical method of induction has been the most used. One snag of this method,

especially when the carcinogenic agent is injected or implanted in pellet form subcutaneously, is that it is difficult or impossible to remove that agent after the cancer has appeared. Thus a beneficial effect on the first tumour to be induced in an animal may be masked by the development of a subsequent tumour. Moreover, attempts to remove a pellet may interfere with the blood supply of a tumour and cause it to regress. Even when it is possible to remove the carcinogenic stimulus, the problem remains; if the original stimulus was large enough to induce cancer in a high proportion of animals at risk, then it would most certainly have been large enough to induce a precancerous state over a fairly wide area, and in such an area subsequent tumours may be expected to arise at various intervals after the removal of the stimulus. Partly for these reasons, and partly because of the difficulty of producing large numbers of animals ready for test all at the same time, testing methods involving the use of induced tumours find limited use at present. It has been necessary, therefore, to take a third step away from the primary problem and consider the use of transplantable animal tumours.

In experiments in which chemical substances, physical agents or viruses are being tested for carcinogenic potential and in which tumours of doubtful malignancy are induced, one of the tests for malignancy has been the ability of grafts of the tumour tissue to grow in isologous or homologous hosts. When the transplanted tumours have grown, subsequent serial transplantations from animal to animal have frequently been undertaken. The test is not always helpful or conclusive, but through its practice a great number of transplantable tumour lines have accumulated, mostly mouse and rat tumours. During the early stages of serial transplantation, tumours tend to retain a recognisable similarity to the original one, and not only may their growth rate be limited, but also they may grow only in some of the hosts into which they are grafted. However, as serial transplantation proceeds, changes take place as a part of what has been termed "progression" (Foulds, 1954). Recognisable features tend to disappear, and the take-rate, and sometimes the growth-rate also, increases. Moreover, whereas the original tumour may have grown only in isologous hosts, after serial transplantation for many generations growth may occur in homologous hosts of entirely different genetic make up. It is this stable undifferentiated and anti-genically "neutral" type of transplantable tumour that is the back-bone for all programmes of testing for antitumour activity. Even so, it would be wrong to consider any transplantable tumour entirely stable. Koller (1960) reported that the average number of chromosomes in the apparently stable transplantable Walker tumour fluctuated between 52 and 64 during ten years of observation.

Large scale testing for any type of drug action may be likened to high-sea net fishing (Bergel, 1960). If the mesh of the net is too fine, many small and unusable fish will be caught. If, on the other hand, it is too open or if its mesh is uneven to the extent of having some large holes in it, then sizable and potentially usable fish may escape. The fear of missing a large fish because of a hole in the net is balanced by the fear that too long spent in freeing the net of small fish will delay the catching of larger ones.

In the United States, screening is organised by the Cancer Chemotherapy National Service Center (C.C.N.S.C.). This organisation sponsors research not just in the U.S.A., but all over the world. It attempts to examine tens of thousands of unselected compounds each year by a variety of approaches. No other country sponsors a screening programme of the same type or on the same scale. Therefore in considering screening of unselected compounds, we have drawn largely on the published experience and conclusions of the C.C.N.S.C. programme (v. below, Section L). For test methods applicable to selected compounds (i.e. compounds expected to have a particular type of antitumour effect, such as alkylating agents or anti-metabolites, or compounds picked out because of apparently beneficial effects seen in the primary tests), on the other hand, we have drawn both on our own experience and on the immense fund of published work from all over the world. These methods are surveyed in Sections C–L below.

The sheer economic impossibility of any other country's attempting something equivalent to the C.C.N.S.C. programme (and the generosity of the C.C.N.S.C. in accepting for test compounds made in other countries) has had the effect of making workers in Britain, at least, much more discriminating in their approach. Careful consideration has been given both to available information and to theories relating activity and chemical structure before new compounds have been synthesised or submitted for test. Bergel (1960) has likened this non-empirical approach to fly-fishing, since information is available about not only the type of fish but also the best methods of catching it.

If we look at the agents generally considered to be useful against cancer in the clinic (v.Table VI, p. 866), we find that neither method has been very successful in recent years. Certainly the hope that the C.C.N.S.C. tests would pick up entirely new classes of tumour-inhibitory compound has had little fulfilment, unless the recent discovery of the phthalanilides (Schepartz, Wodinsky and Leiter, 1962) leads eventually to useful drugs. Such new compounds as have been found belong to classes of agent whose efficacy was already recognised, and most of them have evolved, as might be expected, from the discriminatory approach. This poor record of success does not give us much heart to write enthusiastically about existing test methods. However,

we do so in the hope that, on the basis of more fundamental knowledge and with more rationale, the best of these methods will still find a use in the future.

As indicated above, the transplantable animal tumour is the most important tool, whatever the approach. Consequently a large part of this chapter is devoted to it. The range of transplantable tumours at one time or another used in testing for antitumour activity is enormous; it would be uninformative for us to attempt here to make a comprehensive list of them. Instead, we have tried to mention only the ones that have found most favour or are of special value in testing certain types of drug or are most successful in detecting activity in drugs of known activity or whose use illustrates a special idea or principle, and sometimes those thought specially suitable as models for particular types of human cancer. Little of what we say is new, and for the finer details of methods the reader will be referred to what we consider good, but not necessarily the original, descriptions of techniques.

Of the other test methods described in Sections D–L below none has yet taken pride of place over the transplantable animal tumour tests; indeed the latter, rather than clinical usefulness, are still the yardsticks by which the efficiency of all other methods are judged (Lasagna, 1958). However, since none of our test methods has yet brought to light an entirely efficacious drug, there is a reluctance to abandon alternative methods or the search for new ones.

B. Tests with Transplantable Tumours

The three essential ingredients of tests of this kind are the animals, the tumour and the test substance, in a form in which it can be administered. These will be considered first.

1. THE ANIMALS

The selection of a particular transplantable tumour determines the species and often the strain of animal to be used, but with tumours that grow in various strains it is necessary to select the best one for the purpose of the test. Sometimes the choice may be between a genetically homogeneous line inbred for more than 20 generations, the F1 hybrids of two such lines or a stock outbred strain. In general, outbred animals are adequate for primary tests, but pure line animals, or their F1 hybrids, are preferable for dose–response studies, for determining therapeutic ratios and for comparing the effects of two or more drugs.

Whether pure line, F1 hybrids or random-bred animals are used, it

is obviously essential that a constant check be kept, not only on the "purity" of the pure lines, but also on the randomness of the random-bred animals. For the former purpose, spot-checks for homogeneity should be made at frequent intervals, a convenient one being to establish whether or not skin cross-grafted from one animal to another remains viable over a long period.

Animals of the same sex should be used for any one test.

2. THE TUMOUR

In Sections C and D below, the rationale for choosing particular transplantable tumours is dealt with in some detail. Here we are concerned more with the physical condition, including viability and growth potential, of the tumour material. Transplantable tumours are usually obtained from other centres, either frozen or carried by animals into which they have been transplanted. As a rule it is desirable that a tumour arriving newly at an institution should be passaged for at least three generations before it is regarded as fit to be used for drug testing. For one thing, there are likely to be genetic differences between the animals to which the tumour is accustomed and those at the receiving laboratory, and the effect of these differences may be to cause the tumour to grow more slowly and less regularly at first. Again, it is well known that frozen tumours grow less well for at least one generation after being revived. Even when the animals due to receive the tumour are nominally of the same strain and subline as the donating animal, it is important to remember that a journey, perhaps unusual diet and a change in environment may temporarily alter the growth rate and behaviour, both of the travelling animal and of the tumour material it bears (Griffiths, Hoppe and Cole, 1961; Minster, 1962).

It is advisable to make stringent bacteriological tests on tumour material received from elsewhere. Transplantable tumours seem to constitute ideal growth media for passenger bacteria and viruses alike (Old, Benacerraf, Clarke, Carswell and Stockert, 1961; Moore, 1962; Huebner, 1959). No doubt the poor vascular supply (Goldacre and Sylven, 1959) protects such organisms from the immunological defences of the host. A simple procedure is to put some of the tumour material, at the time of the first passage, for aerobic culture in broth and anaerobic culture in deoxycholate medium and to incubate at 37°C for 48 hr. The culture media should then be inspected after 24 hr. and 48 hr. and examined for organisms in a routine manner. An absence of bacterial growth in such cultures can, however, not be regarded as completely assuring absence of contaminating micro-organisms. It is therefore advisable to isolate not only all animals coming into a unit, but also all animals bearing transplantable tumours derived from elsewhere,

until the tumours have been passaged for at least three generations without the simultaneous passage of an obvious disease syndrome.

3. THE TEST MATERIAL

Testing for tumour-inhibitory activity may occur early or late in the examination of a new substance. Whichever happens, it is essential that all that is known about the substance should be available to those responsible for designing the test. Toxicity, total dosage, dose schedule, solvent and formulation, chemical purity and stability and route of administration should all be carefully considered, and the most suitable transplantable tumours should then be chosen. Biological testing for antitumour activity is a costly procedure; any time spent in discussing and planning tests beforehand can be well spent.

As to the degree of purity of substances submitted for test, it is necessary to weigh the costs of further purification against the costs of biological testing. As long as chemists and biologists alike respect the difficulties and value of each others' work, arriving at a compromise usually presents no difficulty.

The decision to submit a drug for clinical trial is made in the light of the spectrum of transplantable animal tumours against which it is effective and of a therapeutic index (such as the ratio of the minimum effective dose, usually the ED_{90}, to the minimum toxic dose, usually the LD_{10}) seen in these animal tumour tests. For calculating the therapeutic index it is essential that assessment of toxicity be made in animals as similar as possible to those used in the anti-cancer test (in species, strain, age and sex). Even when all these conditions are fulfilled, some argue that the toxicity of a drug may be different in the tumour-bearing animal from that in the normal animal (Larionov, 1962) and that toxicity should therefore be assessed in the tumour-bearing animal. An economical method of combining screening for antitumour activity with toxicity testing is represented in Table V, p. 865.

4. THE EXPERIMENTAL PLAN

Even when the testing of large numbers of substances is reduced to something of a routine procedure, it is desirable that testing each substance be regarded as a separate experiment. For instance, because of many variables (loosely referred to as "biological variation"), no two samples of the same tumour from different animals can be regarded as identical for control or other purposes. From a large tumour, it is usually possible to graft from 30 to 100 animals, depending on the type of tumour and method of grafting. Out of the animals so grafted,

at least three groups must be formed by a random selection method: a test group, a "solvent only" control group and a positive control group (treated with a substance known to inhibit the growth of the tumour). It may also be necessary to set aside some of the grafted animals for carrying on the tumour line. In some circumstances it may be justifiable, from long experience with a tumour, to omit the positive control group.

If several test groups are to be set up, the number of grafted animals required may exceed the maximum that it is possible to graft from one tumour. In the C.C.N.S.C. programme (*Cancer Chemotherapy Reports* **1**, 50, 1959) it is regarded as permissible to use for grafting any number of tumours if they were themselves all derived from a single tumour.

It is always easier to determine retrospectively how many animals should have been used in a particular test. In primary testing or qualitative tests, the number should be the fewest compatible with detecting, at a desired level of probability, the least degree of activity that would be regarded as interesting. In both qualitative and quantitative tests the necessary size of groups depends to a great extent on the variation in response between animals exposed to the same treatment. Clearly, therefore, only experience with a particular tumour and statistical analysis of the results obtained with it can enable one to determine the size of groups to be used.

In the C.C.N.S.C. programme, the numbers of animals to be used have been most carefully considered. The numbers vary with the different tumours and with the number of substances under test at any one time. An example is given in Table I.

5. METHODS OF TRANSPLANTATION

The objective of all transplantation procedures is to introduce into the new host an adequate and sometimes a measured amount of viable tumour tissue at a chosen site without bacterial contamination of the tumour and without depositing tumour cells in other sites. In all techniques, therefore, the principles and practice of asepsis must be rigorously followed, for it is far easier to contaminate a tumour line than to rid it of a contaminating micro-organism. In some centres antibiotics are routinely added to the material for transplantation; for instance, the C.C.N.S.C. permit the use of penicillin and streptomycin in their tests (*Cancer Chemotherapy Reports* **1**, 50, 1959).

Ascitic tumours are usually passaged by the intraperitoneal injection of a suitable volume of diluted ascitic fluid after a cell count of a sample of the fluid. Diluents usually consist of sterile glucose–saline solutions, such as Locke's, Gey's, Earle's and Tyrode. Solid tumours are usually introduced subcutaneously or, less commonly, intramuscularly as cell

suspensions, tumour minces or solid pieces. For the two latter introduction is by trocar and cannula of suitable size. With solid tumours no more than 30 min. should elapse from the time the tumour is removed from the donor until it is implanted into the recipients. Gottfried,

TABLE I

Numbers of animals in controls and in treatment groups for S180, Ehrlich ascites, and L1210 when two or more agents are compared with a single control

No. of agents tested	Total animals needed	Number in each treatment	Number in controls
1	20	10	10
2	31	9	13
3	38	8	14
4	48	8	16
5	58	8	18
7*	68	7	19
8	76	7	20
9	84	7	21
10	92	7	22
11	101	7	24
12	109	7	25
13	117	7	26
14	125	7	27
15	133	7	28
16	140	7	28
17	148	7	29
18	156	7	30
19	164	7	31
20	172	7	32
25*	180	6	30
26	187	6	31
27	194	6	32
28	200	6	32
29	207	6	33
30	213	6	33

* It is not worth testing 6, or 21–24, materials—the same number of animals can give valid results on 7 and 25 materials, respectively.

Number of animals in control and treatment groups for Ca755.

No. of agents tested	Total animals needed	Number in each treatment	Number in controls
1	32	16	16
4	72	12	24
9	132	11	33
16	200	10	40
25	293	10	43

Padnos and Molomut (1963) have described a quantitative technique for tumour implantation, whereby standardised suspensions of cells are incorporated in gelatin sponge and implanted by trocar and cannula. With ascites tumours, an interval of up to 1 hr. is permissible.

The skin and hair of the donor and recipient of the tumour tissues are the most fruitful sources of microbial contamination, and the best aseptic techniques are vitiated if due cognisance is not taken of this. After the donor of the tumour tissue has been killed, we recommend that all the hair from every part of its body be removed by electric clippers and the skin sterilised by soaking it liberally in Wescodyne (a colloidal iodine preparation, from Bengue & Co., Wembley, Middlesex, England). An incision is then made to one side of the tumour, and the skin is dissected back to reveal the tumour. Instruments that come into contact with the skin should not be used for the deeper dissection. Tumour tissue should be carefully transferred to a sterile container. Depending on the method chosen for introduction into the new host, the tumour tissue may then be cut into suitable fragments, or finely minced for implantation by trocar and cannula or reduced to a cell suspension by grinding in a suitable tissue grinder, together with a quantity of sterile Ringer solution, for injection by syringe and needle. Hair should be removed from the skin overlying the point of injection on the recipient animals and the area sterilised (e.g. by Wescodyne). The point where the needle or cannula penetrates the skin should be as remote as possible from the site of implantation. When a large trocar and cannula are used for introducing sizable fragments of tumour tissue (as in the method of passaging Walker rat carcinoma 256 at the Institute of Cancer Research), it is advisable to anaesthetise the recipient animal by ether and to make through the skin a small incision that can be closed by surgical clip after insertion and removal of the trocar and cannula. If the trocar and cannula are used to penetrate the skin, there is a considerable risk of bacterial contamination of the tumour tissue.

Selection of tumour tissue suitable for transplantation requires both skill and experience. With most tumours, whether solid or ascitic, there is an optimal time for passaging the tumour, before which it is too small and after which it is liable to ulceration, necrosis, haemorrhage and infection from within the body. In no circumstances should a tumour showing any of these characteristics (except possibly haemorrhage) be used for grafting. Some transplants of the Walker Rat carcinoma 256 have a tendency to become fibrous and hard; these too are unsuitable for passage.

In some centres, fragments of tissue are taken for histological check at the time of each passage; it is in any event advisable that this be done from time to time. Also fragments of tissue should be tested

for bacterial contamination by the methods described above, preferably whenever the tumour is passaged.

The method of transplantation, by cell suspension, mince or larger fragments, is partly a matter of personal preference and partly dependent on peculiarities of the tumour itself. Tumours passaged by cell suspension tend to take a long time to appear; in general, slowly growing tumours are more variable in growth rate than rapidly growing ones. It is probably wise to follow at first exactly the technique used at the laboratory from which a tumour was obtained, and to modify the technique only when there is good evidence that the modification is an improvement. Satisfactory passage of a tumour through many generations without microbial contamination may be relatively easy or exceedingly difficult, depending on the tumour.

No one tumour should ever be more than one person's responsibility, and passaging should never be relegated to a junior or inexperienced person. Cover for transplanting tumours during sick leave and holiday periods is essential and should be planned in advance if valuable tumours are not to be lost. Whenever possible, arrangements should be made to store ampoules containing tumour tissue at the temperature of solid carbon dioxide or below, though not all tumours will remain viable during storage in this way.

6. DOSE SCHEDULE

In most tests treatment with the test substance is begun on the day after transplanting the tumour. It can be argued that any effect of a substance given at this time may be due to interference with the "establishment" of the tumour in its new host rather than to inhibition of its growth subsequent to establishment; that is to say, treatment may have boosted the immunological defences of the host, hindered the development of a blood supply and so on. That these possibilities are not merely theoretical is supported by the fact that drugs are usually less and less effective the later after transplantation they are given. In testing the effect of melphalan on the Walker tumour, for example, it was found that a single dose of 1·7 mg/kg gave rise to 56%, 81%, 100% and 100% inhibition when given to rats bearing 7-day, 5-day, 3-day, and 1-day tumour implants, respectively.

On the other hand, it is clear from the work of Goldacre and Sylven (1959) and others (Owen, 1960; Schatten, 1962) that drugs may never reach central parts of large tumours because of poor blood supply (Fig. 1). Thus drugs that are effective inhibitors of tumour growth may fail to cause the regression of well-established grafted tumours. It is possible that these larger tumours are the better models for the human cancers that it is our final objective to treat. Even so, in

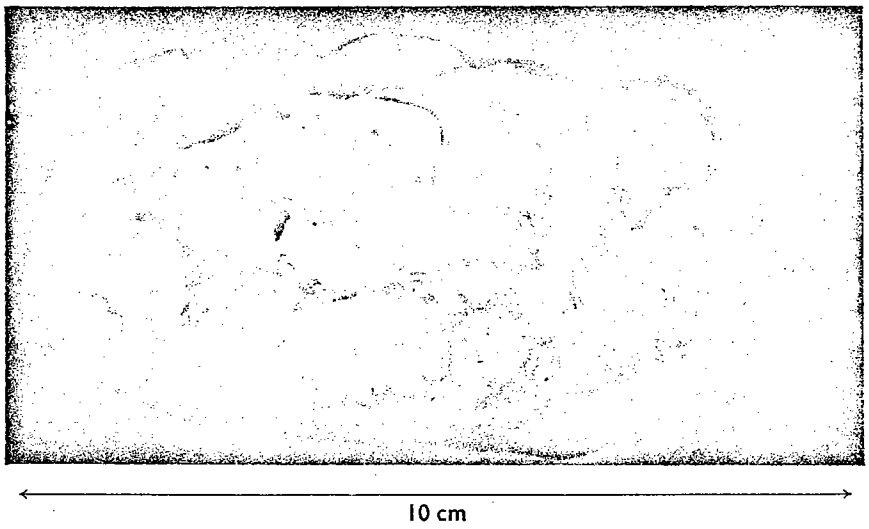


FIG. 1. Walker tumour 13 days after implantation. The rat bearing this tumour was injected intravenously with 4 ml 2% aqueous Lissamine Green 1 hr. before death. Large central areas remain unstained by the dye: these ischaemic regions are known to contain large numbers of viable tumour cells (v. Goldacre and Sylven, 1962, to whom we are indebted for this photograph).

primary testing for antitumour activity it is usual to require sensitive tests, and for this purpose 24-hr. implants are suitable. More stringent tests to see whether antitumour activity detected in the primary test is likely to have clinical value can be undertaken later.

The route of administration and size and spacing of doses will depend on the characteristics of the substance, its toxicity and its expected mode of action. For instance, of alkylating agents it may be sufficient to give a single dose, but of substances expected to interfere with tumour growth because of antimetabolic activity it is usual to give doses once or more than once daily for a limited or unlimited period. Clearly, therefore, biological work of this type requires 7-day per week coverage.

It is desirable that the plan of the experiment should allow flexible dosage. For instance, it should be possible for the man on the spot to reduce or stop treatment on his own initiative when problems of toxicity arise. Dosage should be related to body weight, and animals should be weighed before each dose is given. Ideally a substance should be tested at many dose levels, ranging from non-toxic to lethal, and then no flexibility would be necessary.

It is generally regarded as poor technique to inject the test material directly into solid tumours or near them. It is relatively easy to damage the blood supply to a tumour and to infect it by such procedures, so

that an observed regression may have nothing to do with the nature of the test material. For transplantable tumours grafted into the subcutaneous tissues, therefore, drugs are usually given by intraperitoneal injection, intravenous injection, stomach tube or subcutaneous injection into the opposite flank. With ascites tumours, however, intraperitoneal injection does not damage the blood supply, and provided stringent asepsis is observed this route is permissible.

7. OBSERVATIONS ON TEST ANIMALS

All animals must be weighed at the beginning of the test and thereafter at regular intervals. When test substances are given daily it is convenient to weigh the animals at the time of treatment, since the body weight should determine the size of the dose to be given.

Even when extensive toxicity tests have been undertaken before the antitumour test, unexpected toxic effects may be observed. Weight loss, or failure to put on weight, is the commonest sign of drug toxicity, though it can naturally also occur for reasons unconnected with the test or because of infection or ulceration of the tumour. In tests on transplantable leukaemias and of drugs liable to affect one or more of the elements of bone marrow, regular blood counts should be made. All other toxic effects, such as general malaise, trembling and diarrhoea, should be observed and recorded for direct comparison with animals in the control groups.

With solid tumours it may be necessary to record tumour size measured at two or three diameters by calipers. When this is done it is essential that animals of all groups be measured by the same individual, since people have widely different ideas on the use of calipers.

Not only the size but also the quality of the tumour growth, whether it consists of firm healthy tissue, or is cystic or haemorrhagic, whether it has or is about to ulcerate, and so on, should be ascertained and recorded. In Great Britain, daily observations such as these are necessary not only as a part of the proper conduct of the test, but also because of the desirability and necessity of compliance with the 1870 Cruelty to Animals Act: the fact that an otherwise healthy animal carries a large transplanted tumour does not contravene the provisions of the Act, but if a tumour becomes infected or ulcerated it is necessary under the Act to kill the animal at once.

8. ASSESSMENT OF RESULTS

There are several common ways in which the effect of test substances on the growth and behaviour of transplantable tumours are assessed; they include repeated measurement of tumour size by calipers, weighing

the tumour tissue at a fixed time after transplantation, survival time, the total packed cell volume (TPCV) for ascites tumours and the effect on the circulating white blood cells for transplantable leukaemias. All these methods of assessment depend on a comparison between values obtained for test and control groups. Results are expressed as mean percentage inhibition of tumour growth, the growth in solvent controls being regarded as 100%, or as simple ratios test/control (T/C) or control/test (C/T) or some convenient modification of these; thus, in the C.C.N.S.C. programme L1210 leukaemia (an ascites tumour) is assessed by

$$\frac{\text{Survival time (days) of test animals} - 5 \text{ days}}{\text{Survival time (days) of control animals} - 5 \text{ days}}$$

(a) *Solid tumours*

Accurate measurement of solid tumours in living animals is difficult. Conversion of caliper measurements to tumour weight has been attempted by various authors (Schrek, 1935, 1936a,b). Druckrey (1959) describes a method of calculating the weights of tumour masses by weighing life-size plasticine models of them. The weight of tumour material dissected from an animal at the end of a test is a much more accurately assessable quantity. However, postmortem examination of animals in antitumour tests is a procedure requiring both experience and skill. Obviously it is essential that tumour tissue, as opposed to normal or inflammatory tissue, should be correctly recognised. If there is doubt, as there may be even when an experienced person is responsible for the dissection, sections should be prepared from the tissue in question and the assessment of the result of the test postponed until the histological report is available. Considerable difficulties in interpretation arise where tumours are found to be cystic or to contain large areas of haemorrhage. In these circumstances is the weight of the tumour including cystic fluid and blood the best measure, or should the cysts be drained and the blood washed out before the tumour is weighed? In practice the total weight, including fluid and blood, is the one usually recorded.

(b) *Non-takes*

On occasion tumour-transplants fail to grow in otherwise untreated animals. Such "non-takes" may be indistinguishable from cases in which there has been complete inhibition of growth by an effective drug. When there is a low incidence of "non-takes" among a large control group, it is usual simply to eliminate a similar number of "no growths" from each treatment group before assessment. But if a larger number of "non-takes" is seen among the controls, the whole test must

be regarded as invalid (*Cancer Chemotherapy Reports* **1**, 56, 1959; and **25**, 47, 1962).

(c) *Ascites tumours*

From the study of a number of ascitic tumours, Patt and Straube (1955–56) and others have shown that, provided there is adequate control of the weight and strain of the animal, of the diet and of the number of ascitic cells inoculated, tumour development passes through an initial phase of rapid cell multiplication and then to a period of growth decelerating towards an asymptote. Complicating factors, such as the development of a solid tumour or the nutritional status of the animal, are relatively unimportant during the first phase of rapid growth, and during this period tumour growth may be measured directly by enumerating the total number of free malignant cells in the peritoneal cavity or by measuring the cellular material present (TPCV*).

Thus drug effect may be assessed by comparing tumour growth in treated and control animals for a fixed period of time during the log-growth phase. This, according to Patt and Straube (1955–56), is usually the most satisfactory measure. However with the L1210, a leukaemia grown in the peritoneal cavity, comparison of survival time in treated and control animals is more informative.

The method of enumerating the total number of cells is simply to collect all the ascitic fluid, add to it the product of washing out the peritoneal cavity repeatedly with isotonic saline, mix, and count the cells in a measured portion of the total.

The TPCV is measured by centrifuging at high speed a measured portion of the ascitic fluid for 5 min. The percentage of precipitated cellular material is then multiplied by the difference between the weight of the animal before and after drainage of the ascitic fluid.

Theoretically the TPCV may not reflect the total number of cells if there should be a change in mean cell size. In fact, however, there is usually good agreement between measures of TPCV and total cell number, but not between either of these and cell count per unit volume of fluid, total volume of ascites or ascitocrit (Fig. 2, taken from Sassenrath, Costa and Greenberg, 1961). These methods of measurement may be further refined by differential counts. For instance, the percentage of cells capable of taking up vital dyes or the mitotic index may be measured. However, these should not be used as sole measures of drug effect.

(d) *Leukaemias*

With transplantable leukaemias, repeated white-cell counts, both total and differential, have been used in the assessment of drug effect. Survival time, however, is frequently the more meaningful criterion

* TPCV = total packed cell volume.

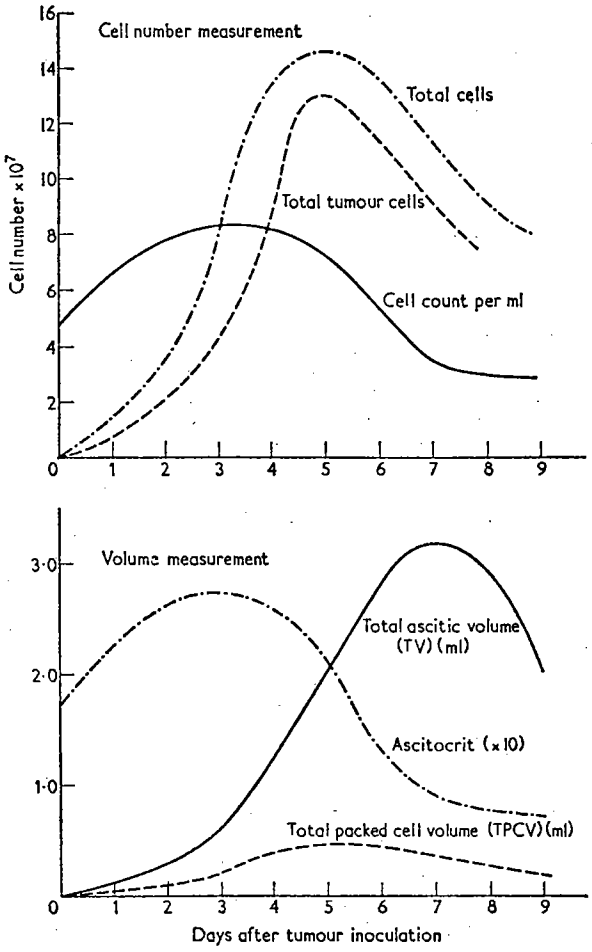


FIG. 2. The curves for total tumour cells and packed cell volume rise and fall together, whereas the cell count per ml total ascitic volume and ascitocrit reach their peaks at different times. (After Sassenrath, Costa and Greenberg, 1961.)

of beneficial drug effect, and a terminal rise in the blood count may serve only to confirm that death was due to leukaemia (Fig. 3).

9. IMPORTANCE OF RECOGNISING TOXIC EFFECTS

Many drugs are tested for antitumour activity at close to toxic levels. It is important both to record the occurrence of toxic effects (as an integral part of their investigation before clinical trial) and also to realise that beneficial effects on tumour growths occur frequently as a result of entirely non-specific toxicity. Tannenbaum and Silverstone have shown in numerous studies (1953, for example) that the

CHESTER BEATTY RESEARCH INSTITUTE, INSTITUTE OF CANCER RESEARCH:
ROYAL CANCER HOSPITAL, LONDON, S.W.3. ENGLAND.

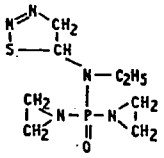
Chemotherapy Screening Tests

FURTH LEUKAEMIA

CB No.:- 40-199

Name of Compound: Thiadazole phosphoramide

Structural Formula:-



Source of compound:- A.T.M.

Date of implantation of leukaemia 21.4.62

Details of implant. 8×10^4 W.B.C. from Rat 62-8

Dose schedule. 10 mg/Kg alternate daily Route. I.P.

Solvent. water

Dates of treatment period. 27.4.62 to 16.5.62 Days of experiment Alternate days from Day 6 to Day 26.

RESULTS

	Treated Group	Untreated Control Group	Positive Control Group (Chlorambucil 12.5 mg/kg on 6th day)
Number of rats	10	10	10
Average survival time (for those dying before 40th day)	24.2 days	15.7 days	25.5 days
Limits of survival time (for those dying before 40th day)	23-27 days	15-16 days	24-26 days
Cures (survival beyond 40th day)	-	-	-
Average wt. of rats at beginning of test	159.4g	162.5g	152.4g
Average weight change up to 2 days before death.	+18.4g	+19.3g	+20.2g
Average white cell count at beginning of test.	10,080	11,840	10,340
Day on which rise in white cell count first noted. (Average for those dying before 40th day)	22nd	10th	23rd

Test carried out by:- K.M. Date:- 20.5.62

Checked by :- F.J.C.R.

FIG. 3. Typical result sheet from use of the Furth leukaemia test. The test substance, thiadazole phosphoramide, administered on alternate days from the 6th day after transplantation of the leukaemia, prolonged the average survival time from 15·7 to 24·2 days, compared with untreated control rats. At this dose level there was no effect on gain in body weight, and the blood count began to rise 2 days before death. The effect seen was similar to, and no better than, that of a single dose of chlorambucil given on the 6th day (i.e. positive control group).

growth of tumours may be markedly affected by nutritional factors. Drugs that adversely affect the nutritional status may seem to inhibit tumour growth. Walpole (1951) reduced the rate of Walker tumour growth by 40% through underfeeding.

Apart from obvious manifestations of toxicity, such as malaise, trembling and diarrhoea, loss in body weight (or failure to gain weight at the same rate as tumour-bearing controls) is the most useful indication of drug toxicity. When an animal has a growing tumour, or is developing malignant ascites, a fall in body weight might be masked by the increasing tumour weight. It is essential therefore that at post-mortem examination not only the weight of tumour tissue be recorded, but also the weight of the carcass after removal of the tumour.

Another possibly useful indication of drug toxicity during a test is afforded by the blood count. Most of the more efficacious antitumour agents tend to suppress mitosis in normal as well as in cancerous tissue, and their effect on bone-marrow proliferation is manifested rapidly as a depression in one or more of the circulating blood elements (Fig. 4).

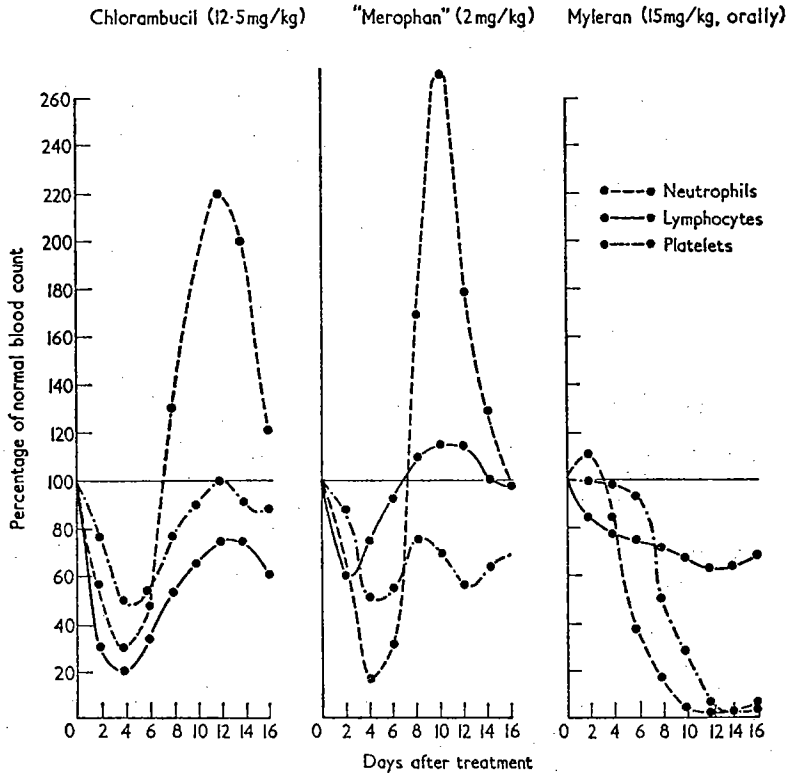


FIG. 4. Patterns of response by circulating blood elements to single doses of myleran, merophan and chlorambucil. Results such as these indicate that drugs may be of special value in particular types of leukaemias.

Finally, we would like to stress the importance of recording all abnormal findings seen at postmortem. Many of these will be attributable to the drugs administered, and the observation may provide not only additional toxicological information and explanations of weight loss or malaise and so on, but also useful leads in other directions.

C. Certain Commonly Used Transplantable Tumours

Almost any malignant tumour, spontaneous or induced, is potentially transplantable within a more or less limited host range. Consequently the literature is full of references to tumours bearing the initials or names of institutes or individuals. It appears to be something of a status symbol to have a transplantable tumour or two to one's credit. However, the true usefulness of a tumour can only be assessed after it has achieved a certain degree of genetic and antigenic stability and its behaviour is consequently reliable and predictable. When there is no special reason to suspect a compound of having a particular type of antitumour activity, it is generally accepted that the best method of detecting tumour-inhibitory action is to submit the compound to a battery of three or more tests against stable transplantable tumours (v. below, Section L). Several surveys have been made of the effects of antitumour agents of proven clinical usefulness on a wide range of transplantable animal tumours with a view to designing the best battery of tests (Suguira, 1962b; Stock, 1960). Perhaps the most important of these was in a report edited by Gellhorn and Hirschberg (1955). Some details of several of the more commonly used transplantable tumours are given in Table II.

D. Transplantable Tumours from Specialised Tissues or Used as Models for Studying Particular Forms of Human Cancer

Many attempts have been made to develop transplantable tumours specially suitable as models for the study of specific types of cancer. For instance, certain melanotic tumours, such as the Cloudman S-91 melanoma (Cloudman, 1941) and the Harding-Passey melanoma (Harding and Passey, 1930), might be expected to be good biological tools in the quest for drugs effective against melanoma in man. At present, considerable interest is centred on the use of transplantable plasma-cell tumours in mice (Potter and Boyce, 1962; Potter and Robertson, 1960) as models for multiple myeloma in man; the fact that abnormal plasma γ-globulins and proteinuria of the Bence-Jones type accompany the murine disease makes this a particularly hopeful

TABLE II

Certain commonly used transplantable tumours

Name of tumour	Species and strain	Origin of tumour	Characteristics and varieties	Methods of transplantation	Method of assessment	Sensitivity to drugs	References to its use
Sarcoma 180 (also known as the Crocker sarcoma)	Mouse Various strains including BALB/C and random-bred Swiss mice	Discovered by W. Woglom, of the Crocker Laboratories at Columbia University, in the axilla of a white male mouse. It was originally described as a carcinoma but after repeated transplantation it took on the characteristics of a sarcoma (Sugiura Stock, 1952)	Solid tumour which grows to a size of about 12 mm diameter in 7 days and occasionally metastasises to lungs. An ascitic form also used. (Sugiura & Creech, 1955–56; Sartorelli et al., 1960; Sassenrath et al., 1958, 1961)	Subcutaneous transplantation of tumour fragments as described in *Cancer Chemotherapy Reports* (**1, 43, 1959; 25, 1, 1962**)	(1) Tumour weight a fixed number of days after transplantation (2) Comparison of growth rates of tumours in treated and control mice; tumour size being measured with calipers	Moderately sensitive to a range of compounds including antimetabolites and alkylating agents	Sugiura & Stock (1952) Sugiura & Creech (1955) Clarke (1955) Stock et al. (1958) Sassenrath et al., (1958) Sartorelli et al., (1960) French & Freedlander (1960)
Carcinoma 755 (also known as adenocarcinoma 755)	Mouse	Mammary adenocarcinoma which arose spontaneously in a C57 black female mouse (Gellhorn et al., 1955)	A slowly growing adenocarcinoma which attains a weight of 3–5 g in three weeks. (*N.B.* Subcutaneous implants do not grow uniformly and the weight at three weeks can vary from 0·1 g to 0 g)	Subcutaneous transplantation as described in *Cancer Chemotherapy Reports* (**1, 44, 1959; 25, 2, 1962**)	Tumour weight, a fixed number of days after transplantation	Extremely sensitive to certain antimetabolites especially those which interefere with purine and pyrimidine synthesis. This tumour has been used to select agents likely to be effective against acute leukaemia in man. (Schabel et al., 1961)	Gellhorn et al. (1950) Gellhorn et al. (1955) Leiter et al. (1959) French & Freedlander (1960) Schabel et al. (1961)
Leukaemia 1210 (L1210)	Mouse DBA strains	Arose originally in a female dba mouse following application of 20-methylcholanthrene to the skin. Subsequently transplanted intramuscularly or subcutaneously (Law et al., 1949–50). Eventually obtained in an ascitic form	Period of rapid growth within peritoneum follows IP injection of leukaemic cells. Dissemination occurs in both ascitic and solid forms, killing animal in 9–12 days. Mean survival time of ascitic forms depends on size of inoculum	(1) Intraperitoneal injection of 10^6 leukaemic cells, as described in *Cancer Chemotherapy Reports* (**1, 45, 1949; 25, 3, 1962**) (2) Subcutaneous transplantation of fragment of the solid tumour into the limb as described by Goldin et al. (1962)	Comparison of mean survival time minus 5 days in treated and control groups	Sensitive to antimetabolites (especially folic acid antagonists), alkylating agents and other classes of compounds	Holland et al. (1958) Leiter et al. (1959) French & Freedlander (1960) Goldin et al. (1962)

Ehrlich ascites	Mouse Various strains	Arose as mammary carcinoma in a stock mouse. Obtained in ascitic form by i.p. injection of tumour emulsion (Loewenthal Jahn, 1932)	Numerous varieties derived from single cells have been used, e.g. 3 ascitic forms: ES (hypotetraploid) (Creech et al., 1955). ED (hyperdiploid) (Sugiura & Creech, 1955–56; Lettre, 1950) Clone EF (hypotetraploid) (Creech et al., 1955) and a solid tumour (Sugiura, 1953) which grows to size of about 12 mm diameter in 14 days. The ascitic forms kill in 11–17 days depending upon strain and size of inoculum. Dissemination occurs at a late stage	Intraperitoneal injection of 2×10^6 malignant cells as described in *Cancer Chemotherapy Reports* (1, 51, 1959)	(1) Total packed cell volume (TPCV) (2) Survival time	Both forms and especially the solid, are insensitive to most agents. Certain alkylating agents and antimetabolites have moderate activity	Sugiura (1953) Creech et al. (1955) Sugiura & Creech (1955–56) Boone et al. (1958) Furst et al. (1958) Sassenrath et al. (1958) Creech et al. (1960) Sartorelli et al. (1960)
$6C_3H$–ED Lymphosarcoma (also known as the Gardner lymphosarcoma)	Mouse C_3H strains	Arose in a female C_3H mouse after treatment with equilin benzoate (Gardner et al., 1944). At first it was transplanted subcutaneously but was eventually obtained in an ascitic form by Klein (1951)	Solid tumour kills animal in 3–4 weeks with diffuse infiltration of surrounding tissues. Ascitic form infiltrates peritoneum and kills in 9–12 days	Injection of a standard quantity of malignant cells	*Ascitic form:* (1) TPCV (2) Survival time *Solid form:* Tumour weight a fixed number of days after transplantation	Fairly sensitive to a wide range of drugs	Klein (1951) Sassenrath et al. (1958) Sassenrath et al. (1961) Henderson & Junga (1961) Heidelberger & Baumann (1958)
Walker adenocarcinoma 256 (also referred to as a sarcoma or carcinosarcoma)	Rat Various strains including Wistar	A mammary tumour found on a stock rat at the Johns Hopkins Hospital in 1929 (Sugiura & Stock, 1952)	Rapidly growing tumour which becomes necrotic and ulcerates after 12–14 days, and kills animals after 21 days. Slower growing strains are known	Subcutaneous transplantation of non-necrotic tumour fragments. Treatment with test materials may begin 24 hr. or 7 days after transplantation. In the latter case the test is referred to as the "Established Walker Test" (v. *Cancer Chemotherapy Reports* **25**, 11, 1962)	(1) Tumour weight after a fixed interval (2) Caliper measurement of tumour size in two or three directions	The one day implant is particularly sensitive to many alkylating agents especially the mustards, but is relatively insensitive to antimetabolites. The established tumour is less sensitive to all agents	Haddow et al. (1948) Sugiura & Stock (1952) Bahner (1958) Sugiura (1955) Tarnowski et al. (1958) Druckrey (1959)

TABLE II (continued)

Certain commonly used transplantable tumours

Name of tumour	Species and strain	Origin of tumour	Characteristics and varieties	Methods of transplantation	Method of assessment	Sensitivity to drugs	References to its use
Jensen sarcoma	Rat Various strains including Wistar, Sherman and Sprague–Dawley	Originated as a sarcoma in the peritoneal cavity of a stock rat (Sugiura & Stock, 1952)	Similar to the Walker adenocarcinoma 256	Subcutaneous transplantation of tumour fragments	(1) Tumour weight after a fixed interval (2) Caliper measurement of tumour size in two or three directions	Shows a similar response to that of the Walker tumour but the established tumour is more sensitive	Druckrey (1959) Sugiura & Stock (1952) Sugiura (1961) Sugiura (1962a)
Yoshida sarcoma	Rat Stock albino	Arose as solid tumour in Scrotum with ascites in peritoneum in rat fed 2–amido-azotoluene (Yoshida, 1949)	An ascitic form grows rapidly for 4 days and then begins to invade the tissues. It kills in about 12 days. The solid form grows at a moderate rate and kills after 30 days	Intraperitoneal injection of a standard number of malignant cells or subcutaneous transplantation of tumour fragments	*Ascitic form* Survival time *Solid form* Caliper measurement of tumour size in 2 or 3 directions	Both solid and ascitic forms even when well established are quite sensitive to certain alkylating agents, antimetabolites and antibiotics	Yoshida et al. (1950) Okubo et al. (1951) Oettel & Wilhelm (1958) Druckrey (1959) Matthies (1961)

TABLE III

Transplantable tumours derived from specialised tissues

Name of tumour	Species and strain	Origin of Tumour	Characteristics and varieties	Methods of transplantation	Method of assessment	Sensitivity to drugs	References to its use
Cloudman S91 melanoma (subline developed by Verdrich et al., 1961)	Mouse DBA strains	Arose spontaneously in a JAX–dba female mouse (Cloudman, 1941). The subline developed by Verdrich et al. (1961) is transplantable to hybrid mice	Subcutaneous implants grow more rapidly than did original S91. Tumours weigh 5–7 g after 28 days and kill animal between 32 and 46 days	Subcutaneous injection of tumour cell suspension into axillary region or left flank (v. *Cancer Chemotherapy Reports* **25**, 7, 1962)	Weight of the tumour on fixed day after treatment	Sensitive to many alkylating agents and some purine analogues. The original S91 is less sensitive (Downing et al., 1953; Luck, 1957; Riley, 1959)	Verdrich et al. (1961)

Harding–Passey melanoma	Mouse Various strains including DBA and Swiss albino	Arose spontaneously on ear of a stock mouse (Harding and Passey, 1930)	Slow-growing intensely pigmented tumour	Subcutaneous transplantation of tumour fragments	Tumour weight: (1) Weight of the tumour on fixed day after treatment (2) Caliper measurement of tumour size in two or three directions	Completely insensitive to most compounds. Inhibition of this tumour has only been seen with melphalan, thio-TEPA and mitomycin C	Sugiura & Stock (1952) Luck (1956) Sugiura (1961)
Glioma 26	Mouse C57 black	Originated in a C57 black mouse after intracerebral implantation of 20-methylcholanthrene (Zimmerman & Arnold, 1941)	Solid tumour with characteristics of glioblastoma multiforme. It grows slowly without ulceration and kills animals after about 10 weeks	Subcutaneous transplantation of tumour fragments	Weight of the tumour on fixed day after treatment	Inhibited by certain antimetabolites and alkylating agents	Gellhorn et al. (1954–55) Gellhorn et al. (1955) Sugiura (1961)
Fortner intestinal adenocarcinoma	Golden syrian hamster	An adenocarcinoma which arose spontaneously in the wall of the small intestine of a Golden syrian hamster (Fortner Gale, 1958)	Fairly fast-growing solid tumour which frequently metastases to lungs and lymph nodes	Subcutaneous transplantation of tumour fragments	Measurement of tumour size with calipers in two directions	Insensitive to most agents. Inhibition has been observed with 5-fluorouracil and 5-fluorouridine	Sugiura (1961)
Ridgway osteogenic sarcoma	Mouse AKR	A spontaneous tumour first observed in 1948 as an inguinal mass in a male AKR mouse. Histological examination revealed the presence of cells with foci of bone formation (Sugiura Stock, 1952)	After repeated transplantation this tumour has lost its bone structure but retains a high alkaline phosphatase activity. It reaches a size of 12–14 mm diameter in 14 days	Subcutaneous transplantation of tumour fragments	Measurement of tumour size with calipers in three directions	Fairly sensitive to many compounds	Sugiura & Stock (1952) Sugiura (1961)
Plasma cell tumours (including several types described by Potter and Fahey (1960) e.g. the X5563 plasma cell neoplasms	Mouse C3H/HE and other strains	The first of these arose in the ileocaecal region of a 22-month-old female C3H mouse gonadectomised at 2 months (Potter et al., 1957)	Most varieties are fairly slow growing, and only disseminate at a late stage. They resemble human multiple myelomas in their histology, in the development of osteogenic bone lesions and in the production of abnormal serum globulins	Subcutaneous transplantation of tumour fragments	(1) Survival time (2) Measurement of tumour size with calipers (3) Correlation of tumour inhibition with level of abnormal serum globulin	Sensitive to many known antitumour agents	Hayes et al. (1962) V. Rosenoer & M. Whisson, personal communication

proposition (Hayes, Spurr and Hines, 1962; V. Rosenoer and M. Whisson, personal communication).

In general, however, there is one objection to this approach: the special characteristics of tumours tend to be lost during the early passage generations; by the time the tumour has achieved genetic and antigenic stability it is frequently little different in its characteristics and behaviour from tumours originating in completely different tissues. This is not always so; sometimes recognisable characteristics (morphological and biochemical) are retained indefinitely. However, even when the morphological characteristics are retained (as, for example, in glioma 26), there is not necessarily a good correlation between results of tests and clinical usefulness.

With the drive, now gaining momentum, to study the biological and biochemical characteristics of human tumours (Heidelberger, 1961, *Cancer Chemotherapy Reports* **20**, 1, 1962; Annotation in the *British Medical Journal i* 1752, 1961), there is hope that particular human cancers can be matched with transplantable tumours on the basis of their enzyme contents and other biochemical features. In Table III is given a brief list of transplantable tumours thought to be specially relevant to certain human situations.

E. Tests for Activity against Spontaneous Tumours

The difficulties of this type of test have been pointed out above, as also has the fact that most spontaneous tumours arising sufficiently regularly to provide the basis for a test technique are virus-induced (v. Section A above). A third difficulty is that only spontaneous tumours readily recognisable at an early stage in the living animal are suitable for test. The choice of suitable spontaneous tumours is therefore limited, and in practice only a few tumours of this type have been used. Most of them are mammary tumours (v. also Section F.2 on virus-induced tumours). As long ago as 1924 Nakahara (1922, 1924, 1925) reported investigations into the effects of certain unsaturated fatty acids on spontaneous tumours in mice. Woolley (1953) used spontaneous mammary tumours in three strains of mice (Swiss, C. and C3H) and observed transient, partial or complete regression of some but not of all tumours in response to treatment. Scholler, Philips, Sternberg and Bittner (1956), Scholler, Bittner and Philips (1957) and Scholler and Bittner (1958) found that spontaneous mammary adenocarcinomas in mice are resistant to chemotherapeutic agents that are effective in first- and second-generation transplants of the same tumours. The slight regressions they observed in mice bearing spontaneous tumours could have been entirely due to the non-specific toxic effects of the treatments

given. Clearly, such tumours provide a stringent test of antitumour activity and should not normally be used for primary tests.

F. Tests for Activity against Induced Tumours

The objections to the use of transplanted animal tumours (such as host immunity, progression and restricted blood supply) have led to attempts to use chemically or virally induced tumours. Some of the difficulties, both practical and economical, in the use of such systems have been mentioned in Section A above.

1. chemically induced tumours

Carcinogenic polycyclic hydrocarbons (such as 3,4-benzopyrene, 20-methylcholanthrene and 1,2,5,6-dibenzanthracene and their derivatives) have been used to induce tumours for test systems. Wodinsky, Kensler and Leiter (1961) were able to induce fibrosarcomas in 80% of weanling Swiss mice within 10 to 15 weeks by subcutaneous implantation of pellets containing 3,4,9,10-dibenzopyrene. Animals with tumours between 3 mm and 10 mm in diameter were randomly assigned to test and control groups, and the effect of drugs on tumour growth were assessed by caliper measurement. They saw some growth inhibition from daily drug treatment with certain compounds. At the Chester Beatty Research Institute, spindle cell sarcomas are induced in Wistar rats by the subcutaneous implantation of pellets containing 3,4-benzopyrene. A high incidence of tumours is seen after 6 – 8 months, and the effect of drugs on tumour growth is assessed by repeated caliper measurements. Marked tumour inhibition is rarely seen in this test. Baker and Tregier (1962) have assessed the effect of drugs in delaying the appearance of tumours in mice treated with 3,4-benzopyrene. Woodhouse (1947) has used mouse epitheliomas induced by cutaneous application of 3,4-benzopyrene in acetone and assessed drug activity by measurement of tumour size at the base before and during treatment.

Sarcomas induced by 20-methylcholanthrene have been studied by Merker, Baba and Singer (1960) and by Owens and Busch (1961); leukaemia induced by the same substance was used by Block and Takano (1952), who judged tumour-inhibitory effect by differential white cell count and survival time.

2. virus-induced tumours

The two most commonly used virus-induced tumours are the Friend leukaemia and the Rous sarcoma. These will be considered separately.

(a) *Friend leukaemia*

This tumour was first seen in adult Swiss mice by Friend (1957), who found it to be serially transmissible by injection of cell-free filtrates derived from leukaemic-spleen homogenates. Suguira (1959, 1961) used this tumour for drug testing. Inhibition of splenic weight gain, reduction in titre of viable virus (assessed by bioassay) and prolongation of survival time were used as measures of inhibitory effect. Of these, the first is somewhat laborious and the second most time consuming, for there is a 2–4 month interval between inoculation of the virus and appearance of leukaemia. Suguira's (1959) results are interesting in that there is no correlation between them and the results of other tests. Thus the Friend leukaemia was inhibited by some alkylating agents, some antimetabolites and some antibiotics (myleran, 6-mercaptopurine, mitomycin C), but unaffected by others (HN2, amethopterin, actinomycin D). Most other tumours (such as the Walker adenocarcinoma 256 and adenocarcinoma 755) show a tendency to be sensitive to most or all the members of one class of agent (for example, alkylating agents and antimetabolites). Because of its unique behaviour in this respect, the Friend leukaemia must be regarded as potentially useful, since it may detect activity that might otherwise be missed.

(b) *Rous sarcoma*

This virus-induced sarcoma was first described by Rous in 1911. Being an easily measurable local tumour, it is easier to use in testing than the Friend leukaemia. However, its marked insensitivity to all classes of chemical agent so far used, both in local tumour growth and survival time, has not encouraged many workers to use it. Induction of the tumour in young chicks is by implantation of tumour fragments or inoculation of cell-free material prepared from tumour homogenates. Experimental details for the use of this tumour as a test technique are given by Suguira (1962a), Bather (1960) and Pienta, Bernstein and Groupé (1961).

G. Tests on Heterotransplanted Human Tumours

In general, the greater the antigenic difference between the animal from which a tumour is derived and the animal into which it is transplanted, the less readily will it grow and the easier it is to interfere with its growth. Except in special sites protected from the immunological defences of the body (such as the anterior chamber of the eye and the cheek pouch of the hamster) or under conditions in which the immunological defences of the body are paralysed (for example, after neonatal thymectomy, high doses of X-rays or cortisone treatment), tumours are not transplantable from one species to another. Use has

been made of these exceptions to test human cancer tissue for sensitivity to antitumour agents. Heterotransplantation into the hamster cheek pouch and into cortisone-treated animals of various species have been undertaken fairly extensively.

1. THE HAMSTER CHEEK POUCH

This site was first used for tumour transplantation by Lutz, Fulton, Patt, Handler and Stevens (1951), the tumour transplant being of hamster origin. Shortly afterwards (Patt, Handler and Lutz, 1951, and Handler, Patt and Lutz, 1952) heterologous tumours were shown to grow in the pouch, and the way was open for a direct test with human cancer tissue. A description of the techniques involved and of the results obtained up to 1958 is given by Handler (1958) and by Toolan (1958). Since 1958 the test system has become more standardised, and the number of human tumour lines in common use is small. Moreover there is an increasing tendency for cortisone to be used to condition the host. Woolley (1962) reported the results of testing 19 drugs of known clinical antitumour efficacy against three human tumour lines, H.S. No. 1 (a sarcoma), A-42 (a bronchogenic carcinoma) and HEp No. 3 (an epidermoid carcinoma) in the cheek pouch of cortisone-treated hamsters. Whether such serially transplanted human tumours bear a close relation to the cancers from which they were originally derived is naturally open to considerable doubt, though Toolan (1958) reports no loss of antigen in some such tumours.

2. CORTISONE-CONDITIONED AND X-IRRADIATED ANIMALS

There is now an extensive literature on the use of cortisone and X-irradiation for conditioning animals so that they may support the growth of tumour tissue of human origin. Not surprisingly, many are concerned about the artificiality of such test systems, since not only is the antigenic difference between tumour and host likely to change the growth of the response of the tumour to administered drugs, but so also may the continued administration of cortisone.

A short history of the use of X-irradiation and cortisone for conditioning purposes is given by Toolan (1958). On account of the difficulties of arriving at a suitable conditioning dose of X-irradiation (because of wide variations in the sensitivity of different species and strains), the development of readily usable heterotransplantation techniques of this kind came about only after the discovery (Toolan, 1953, 1954) that cortisone could prolong indefinitely the suppression of immunological defences initiated by X-irradiation. At the present time, according to Toolan (1958), the optimal method of conditioning

animals varies with the species: for the hamster and the rabbit cortisone alone is preferable, for the rat a combination of cortisone and X-irradiation treatments is most effective, and for the mouse either cortisone alone or in combination is suitable.

The technique and difficulties of using conditioned rats for screening purposes are described by Teller, Merker, Palm and Woolley (1958a), Marsh and Cullen (1958), Palm, Teller, Merker and Woolley (1958), Teller, Merker, Palm and Woolley (1960) and Teller, Palm, Merker, Harris and Woolley (1958b); and the results of testing 19 compounds of known clinical performance in this system are described by Teller (1962).

The technique for mice is described by Gallily and Woolley (1958) and Takayama and Woolley (1958). After testing 19 compounds of known clinical efficacy against human tumours grown in conditioned mice, Merker, Anido, Sarino and Woolley (1962) concluded that so far there is no evidence that this test system would detect any useful effect missed by simpler transplantation techniques.

3. EMBRYONATED EGGS

The ability of heterologous tumours to grow on the chorioallantoic membrane of the chick embryo was first recognised by Murphy (1912). Harris (1958) gives a brief survey of the development of the technique to the point at which Dagg, Karnofsky, Stock, Lacon and Roddy (1955) first grew tumours of human origin in eggs and tested substances for antitumour activity on them. The same author (Harris, 1962) has recently reported the results of testing 19 substances of known efficacy in the clinic in this system. It appears that this relatively simple and inexpensive technique is capable of detecting useful activity in most of the 19 substances.

H. Tests with Tumour Cells Grown in vitro

Recent rapid advances in tissue-culture technology have made three different kinds of in vitro tests possible.

Thus, (a) drugs of unknown antitumour potential may be tested against cells derived from non-human tumours; (b) drugs of unknown antitumour potential may be tested against human tumour cell lines; (c) drugs of known antitumour potential may be tested against human tumour samples removed at operation or by biopsy.

Tests of the first two types can be reduced to a routine procedure. Moreover, because the nutritional requirements of the cell lines are well known, tests may be made under conditions of prolonged cultivation. Such tests can easily be repeated, and so quantitative studies can

be undertaken. The test of the third type is still in the early stages of development. Only a proportion of the tumour samples grow at all, and few of these can be kept going for more than a few days or for more than one generation. It is therefore difficult to repeat such tests or to get more results to supplement those obtained in the first test.

The central problems common to all cell-culture tests lies in the assessment of the effects seen. First, a suitable control must be found; secondly, a method of measuring the effects of drugs on test and control cultures has to be developed.

Eagle (1958) commented that "the basic question . . . as to whether . . . there are constant and reproducible differences between cells deriving from normal and malignant tissue . . . has not yet been resolved". Biesele (1954) used embryonic mouse skin fibroblasts as a control in testing drugs for effect against sarcoma 180 cells. With some agents, but not with others, there was an inhibition of mitosis in the sarcoma 180 cells but not in the control cells. However, his experiences with this technique during the next 4 years led him to conclude ". . . this differential inhibition of mitosis in culture is not a completely reliable indicator of what will happen when the same agent is applied to the same tumour in an animal" (Biesele, 1958).

In tests of some types no attempt is made to discover whether the toxic effects of drugs are selective for cancer cells as opposed to cells of normal origin. Instead, the comparison is between cultures containing serial dilutions of the test agent. The results so obtained may be viewed in the light of general information about toxicity obtained from in vivo tests, and some kind of in vitro/in vivo therapeutic index is calculated.

Several different measures of the effects of drugs on tumour cells grown in vitro have been used. Of these the most satisfying is, perhaps, bioassay. In well characterised systems, the number of viable cells necessary to achieve "a take" in an animal into which they are transplanted is fairly constant. Thus the "take rate" can be used to measure the number of living cells after exposure to a test agent (Tarnowski and Bates, 1961). Alternatively, when cells are grown in a monolayer on a coverslip, a direct count of viable cells (which remain adherent to the glass) can be made by means of a Chalkley eyepiece (Curtis, 1960; Ambrose, Dudgeon, Easty and Easty, 1961).

A method that appears to be achieving popularity was developed by Miyamura (1956). An agar plate in which the cells are growing is exposed to test agents contained in porcelain cylinders. After a limited period of incubation, the cylinders are removed, and the plate is impregnated with methylene blue. Where the growth of cells has been inhibited, the plate remains blue; where cells are growing the plate is decolourised.

Several other tests for viability of tissue culture cells dependent on the use of dyes have been described. Fujikawa (1959) assessed viability from the response to Feulgen reagent, neutral red, trypan blue and congo red; Yamamoto, Nishioka, Osa, Sakurai, Yoshida and Kikugawa (1959) used 2,6-dichlorophenolindophenol reduction to ascertain the percentage of living cells; Abe, Miyaki, Mizuno, Narita, Takeuchi, Ukita and Yamamoto (1959) used determination of dehydrogenase activity as a measure of cell death in testing 1300 compounds for antitumour activity.

Cobb (1960) used cytological criteria of cytotoxicity for evaluating drug effect; these he lists as granulation, pyknosis, cytolysis, increased cellular debris, loss of staining capacity, nucleolar changes, mitotic reductions and chromosomal aberrations. Overall cytolysis was rated at ++++ and lesser degrees of destruction from +++ to +.

When cells are grown in an agar layer, the number of cells can be estimated by measuring light transmission through the medium (Schuurman, Duncan and Olson, 1960). A method of assaying cell growth was described by Oyama and Eagle (1956). In this method a phenol reagent (Folin-Ciocalteau) is used, and the protein contents of the washed tissue culture cells are measured colorimetrically against bovine albumin solutions as standards. Where facilities for measurement of radioactivity are available, the uptake of tritiated thymidine may be used as a sensitive measure of cell growth in tissue culture.

Typical of the many reports on the use of cell cultures from animal tumours in antitumour tests are those by Biesele (1954, 1958); the use of this kind of test has recently been reviewed by Merchant and Eidam (1961). Similarly tests with stock lines of human cells, such as HeLa, J-111, and HEp 3, are described by Eagle and Foley (1956, 1958), McAllister, Grunmeier, Coriell, and Blakemore (1959), Blakemore, McKenna, McAllister and Coriell (1960), Toplin (1959), Cobb (1960), and Wright, Cobb, Gumport, Golomb and Safadi (1957).

Perhaps one of the greatest difficulties in the use of either animal or human stock cell-lines is the ease with which such lines become contaminated with passenger viruses (Eddy, Grubbs and Young, 1962). So far, little is known about the possible effects of such contaminants on the response of cultures to drugs, but clearly there is plenty of scope for an oncolytic virus (Bennette, 1960) to interfere with the functioning of this type of test system.

The whole subject of the experimental evaluation of potential anticancer agents by in vitro cell culture has recently been reviewed by Dixon, Schabel, Skipper, Dulmadge and Duncan (1961).

Tests for the sensitivity of human tumour material obtained at operation or by biopsy to a range of known anticancer agents have

been used for several years. Studies of this kind are reported by Antikajian, Wright, Plummer and Weintraub (1951), Cobb (1955), Cobb and Walker (1958), and Easty, Wylie and Yarnell (1961); the subject was reviewed by Hirschberg (1958). Cobb, Walker and Wright (1961) report on their experience of this technique over a 5-year period. Each of 196 malignant neoplasms was exposed to a range of chemotherapeutic agents for 96 hr. Of the specimens 35% had to be rejected, either because of failure to grow or because identifiable tumour cells were absent. The authors list the criteria they used for the identification of various tumour cells. They found that different drug types gave rise to different cytotoxic changes and that multiple biopsy specimens from the same tumour (taken at different times) differed in their responses to drugs. At the end of their review they concluded "Cautious clinical extrapolation from tissue culture data is in order pending additional culture-clinical correlation results". Ambrose, Andrews, Easty, Field and Wylie (1962) held that this type of testing may be particularly relevant when it was planned to treat patients by regional perfusion, since this form of treatment brings drugs into direct contact with tumour cells in a way somewhat similar to that occurring in tissue culture tests in vitro. Wright et al. (1962) have recently reviewed this whole subject.

J. Microbial Systems

The desire for quick, quantitative and inexpensive tests for anticancer activity has led many workers to develop and use microbial systems. It has been argued that, if tests of this kind, despite their remoteness from their final objective, namely effect on cancer in man, could have picked out interesting agents, their use would have been justified. As discussed in Section N below, the concept of cancer as analogous to a microbial disease is entirely, or almost entirely, false. Nevertheless it may be argued a priori that, in so far as growth and multiplication of microbes and cancer cells (and normal cells, unfortunately) share similar synthetic pathways, agents that interfere with the growth of one are likely to interfere with the growth of all. Thus it is not surprising that correlations between antimicrobial and antitumour effects have been most evident with antimetabolic agents (Burchenal, Bendich, Brown, Elion, Hitchings, Rhoads and Stock, 1949; Clarke, Philips, Sternberg, Elion and Hitchings, 1953; Gellhorn, Engelman, Shapiro, Graff and Gillespie, 1950). Otherwise the correlation between antimicrobial and antitumour activity is not good (Reilly, Stock, Buckley and Clarke, 1953). In 1955 a panel of investigators under Gellhorn and Hirschberg concluded "there is no evidence for the existence of any non-tumour system which could replace a tumour system as a tool for carcinostatic agents". For antimicrobial systems this conclusion

was based on the experience of 75, —60 with bacteriophages, 7 with bacteria and 8 with fungi. Apart from the microbial tests considered by Gellhorn and Hirschberg and their associates, many other systems involving other species of bacteria and fungi, viruses and protozoa have been studied. However, experience of their use in no way modifies the conclusion of Gellhorn and Hirschberg (1955) quoted above.

A microbial test of special rather than general interest is the one with *Lactobacillus casei* described by Hitchings, Elion, Falco, Russell, Sherwood, Marion and Vanderwerff (1950). This system can be used to detect antimetabolites acting at a particular stage in the synthesis of nucleic acids. The *Tetrahymena geleii* test developed by Kidder and Dewey (1949) can similarly detect specific inhibitors of guanine formation.

Thus microbial systems have been useful in detecting specific forms of anti-metabolic activity and have acted as guides in the synthesis of potent anti-metabolic agents before true antitumour testing, but they have had little value in predicting clinical usefulness.

K. Miscellaneous Test Systems

1. Biochemical Tests

From time to time biochemical methods of testing for antitumour activity have been proposed. We think it wrong even to consider such techniques as equivalent to, or as potential substitutes for, more realistic methods. It is better to regard them as pre-test procedures. Some of the methods suggested are merely tests for anti-growth or anti-metabolic activities of particular compounds based on measurements of the uptake of isotope-labelled precursors into cell proteins and nucleic acids and their precursors (Davidson and Freeman, 1955; Le Page and Greenlees, 1955; Heidelberger and Keller, 1955). Other methods are based on the Warburg hypothesis, according to which there is in cancer cells, as opposed to normal cells, an emphasis on anaerobic glycolysis, and test the effect of drugs on respiration and glycolysis (Murphy, Zipf, Parrish and Katchman, 1961; and Merker, Kimoto, Pearle and Anido, 1961) or the inhibition of glycolysis and fructolysis (Yushok, 1958).

The observation that lactic dehydrogenase activity reflects tumour growth-rate (Riley and Wroblewski, 1959) has led to in vitro tests in which dehydrogenase activity is the criterion used to measure antitumour activity (Shabad, Laginov and Volfson, 1961; Navashin, Fomina and Terent'eva, 1960; DiPaulo, 1962; Jaenner and Krumme, 1960; and Valyi-Nagy, Hernady, Szabo and Jeney, 1961).

Testing a series of pyrimidine analogues, Tiunov (1960) found a correlation between inhibition of the growth of sarcoma 180 and

inhibition of xanthine oxidase activity and suggested tests based on assessing the degree of inhibition of enzyme.

2. CARCINOGENICITY

According to the Haddow hypothesis, there is a relationship between carcinogenicity and tumour-inhibitory effect. It has therefore been customary within the Institute of Cancer Research to test known carcinogens for tumour inhibition and vice versa. However, since tests for carcinogenicity are certainly more arduous than those for tumour inhibition, the former cannot be regarded as a suitable preliminary to the latter.

3. MUTAGENICITY

Tests for mutagenicity have also been suggested for anticancer activity. Using 4 genes in *Escherichia coli*, Hemmerly and Demerec (1955) tested 27 compounds, including several known anticancer agents, but obtained evidence of potent mutagenic effects from only two of them. Schultz, Rothman and Aronson (1955) claimed that their results with *Drosophila melanogaster* justified the conclusion "that the methods used constitute an acceptable procedure for the routine screening of compounds of biological interest". Nevertheless, by their tests they would have missed several agents clinically no less useful than others that gave positive results; more important, numerous false positives would have come to light.

4. TESTS ON DEVELOPING EMBRYOS

Bieber and Hitchings (1955) examined the effects of 27 compounds on the frog embryo, but found little correlation between the results and those from other systems. Results with the chick embryo (Hoshi and Hata, 1958; Karnofsky and Lacon, 1962; Karnofsky, 1955) have been somewhat more interesting, but certainly do not suggest that it can be used in primary tests.

5. THE POSSIBILITY OF TESTS WITH *Dicolyostelium disordem*

Hirschberg and Merson (1955) reported that the effects of 25 compounds on the aggregation and culmination (which are indices of differentiation and morphogenesis) of this slime mould did not "provide a useful guide for the selection of compounds as potential antitumour agents".

6. THE TEST PROCEDURE OF GARATTINI AND PALMA

Garattini and Palma (1961) described a procedure including the quantitative analysis of drug effects on liver regeneration after partial hepatectomy. The complete procedure includes also tests on Walker tumour growth and on agar-granuloma growth. Variables considered are body weight, erythrocytes, leucocytes, liver lipids, liver glycogen, spleen weight and intestine weight and amounts of intestinal DNA, serum lipids, proteins and glycoproteins. Each compound is studied at three dose levels representing constant fractions of a subacute LD_{50} (6 days). The authors, commenting on their experience of the test so far, write "Although it is too early to draw any conclusion, before many other compounds, with known antitumour activity, have been evaluated, it is evident from the data reported that the drugs tested look quite poor so far as the specificity of their action is concerned".

7. INHIBITION OF THE IMMUNE RESPONSE AS A TEST FOR ANTITUMOUR ACTIVITY

Berenbaum (1962) suggested a test of this nature. In preliminary experiments he found that 12 out of 13 antitumour agents significantly reduced the titre of antibodies to TAB vaccine nine days after its injection. The test drug was injected on the second day. In contrast, none of 16 miscellaneous agents, including certain anti-bacterial substances, caused any reduction in antibody response. On present evidence this test must be regarded as promising.

L. Tests for Inhibition of Metastasis

The ability of tumours to undergo metastasis is the most important single factor in rendering them inoperable and fatal in outcome. Accordingly, several workers have tried to develop tests for the inhibition of metastasis. In the natural course of events metastasis is a highly variable phenomenon, dependent on certain chance events (such as erosion of a particular lymphatic or blood vessel) and on an apparently infinite variety of tumour–host relationships. It could therefore be assumed that a satisfactory test for the inhibition of metastasis would involve the use of a completely homogeneous strain of animals, a completely stable transplantable tumour and precisely defined methods of inoculation of tumour cells. Among the methods of inoculation favoured has been the intravenous injection of known numbers of cells as single-cell suspensions. In techniques of this kind the ratio of visible discrete metastases to the number of injected cells has always

been low, usually less than 10^{-4}. Both the viability of the injected cells and the inability of some sites to support the growth of the tumour cells that have come to rest in them may have contributed to the discrepancy. However, active resistance, possibly in the form of a tissue antigen–antibody response, seems also to play a part, since in some circumstances at least the injection of a mixture of living and dead tumour cells is more effective in producing metastases than that of the same total number of living cells (Baserga, Putong, Tyler and Wartman, 1960). The suggested explanation of this phenomenon is that the dead cells attract the antibodies more avidly than the living, so that the latter are less restrained and able to gain a foothold.

No entirely satisfactory techniques for testing ability to inhibit metastasis have yet been developed. Even when the difficulties mentioned above have been sufficiently overcome for regular results to be obtained in untreated control animals, the method of introducing tumour cells bears little relation to the far more usual method of dissemination through the lymphatic system. Attempts to develop testing techniques of this kind are described by Aoki and Fukuoka (1959) and Sato (1959, 1961).

Perhaps the most important fact so far learned from the use of these techniques is that certain of the growth-inhibiting type of cancer chemotherapeutic agents increase rather than decrease metastasis; inhibition of the tissue's immune mechanism has been put forward as the explanation (Kendo and Moore, 1961).

M. Multiple and Sequential Tests

As emphasised in Section A above, there are two basic approaches to drug testing—the small scale discriminatory approach involving the careful examination of selected compounds and the large scale indiscriminate examination of unselected compounds. It should be clear from what was said there that the two approaches are in some ways complementary.

Here we now describe briefly the primary and subsequent tests used in the C.C.N.S.C. programme, and also, as examples of the more discriminatory approach, the screens used at the Institute of Cancer Research in London and the Institute of Experimental and Clinical Oncology in Moscow. Finally we refer to batteries of tests intermediate in approach between the completely undiscriminating and the highly discriminating.

The primary animal tumour test of the C.C.N.S.C. for unselected compounds involves the use of three mouse transplantable tumours, the sarcoma 180, leukaemia 1210 and the carcinoma 755 (or alternatively the Ehrlich ascites). After intensive investigations, these three

tumours were chosen because it was found that together they were capable of detecting all compounds known to be active and to produce the fewest false leads. With these selected tumours a sequential analysis technique is used. This technique is statistically designed to permit 1% of materials to pass an initial test. In the first instance a compound is tested against all three tumours at the maximum tolerated dose (determined on mice bearing the S180 tumour). If the compound exhibits a sufficient effect on comparison of treated with untreated control animals, such as inhibition of tumour growth or prolongation of survival, it is retested a second and even a third time (*Cancer Chemotherapy Reports* **25**, 51, 1962). Compounds that continue to show promise in second and third tests are then studied in more detail and in biological systems of a wide variety. Further studies include determination of the dose–response relationship of the compound against the tumour and secondary evaluation under ten heads.

1. Dose-response tests on sarcoma 180, carcinoma 755, Leukemia L–1210 and Ehrlich ascites, by both oral and parenteral routes.
2. Optimum dosage regimen and dosage forms.
3. Tissue culture tests in fluid or semifluid media.
4. Evaluations against selected animal tumour systems (spectrum).
5. Microbiological evaluations with "wild" and drug-resistant micro-organisms.
6. Cross-resistance studies with drug-resistant tumours.
7. Tests for collateral sensitivity with micro-organisms and tumours.
8. Dietary restriction studies.
9. Combination chemotherapy.
10. Biochemical evaluations on respiration, specific enzyme inhibition, etc.

At the Institute of Experimental and Clinical Oncology in Moscow new compounds are tested against a somewhat variable range of transplantable tumours. For this purpose 11 mouse and 10 rat tumours are available. In addition, rats bearing sarcomas induced by the implantation of 9,10-dimethyl-1,2-benzanthracene are used. After a siting toxicity test, each compound is tested at multiple logarithmically spaced doses on tumour-bearing animals (Larianov, 1962).

At the Institute of Cancer Research in London, for several years special interest has centred on alkylating agents. With few exceptions the Walker rat tumour is sensitive to agents of this type. The anti-tumour activity of agents such as chlorambucil and melphalan was originally detected by injecting groups of six rats bearing 24-hr. old Walker tumour implants with close to maximum tolerated doses of these compounds and comparing the subsequent growth of tumours with that in untreated tumour-bearing controls. After obtaining in the initial tests strikingly positive results, confirmatory tests, tests

with lower doses and tests on "established" tumours (7-days after implantation) were undertaken. Next the drugs were tested against other tumours, such as the sarcoma 180, the Furth leukaemia, the benzopyrene-induced sarcoma and the osteosarcoma 177. Finally, after consideration of all the results, including those from other centres to which the drugs had been submitted for examination against yet other tumours, by tests of other types and after extensive pharmacological examination, a decision was made to try the drugs in the clinic. It should be emphasised that in the Institute the testing of a drug is considered and planned at each stage, and that there is no truly routine procedure.

The Institute is less well equipped to investigate biologically drugs with types of action other than alkylation. Hence compounds of these other types are usually sent to the C.C.N.S.C. or elsewhere for testing at the same time as they are tested within the Institute. The tests currently used at the Institute are shown in Table IV.

We are currently investigating what we hope will be a more economical method of testing drugs for general toxicity and antitumour effect at one and the same time. For this purpose, after a siting toxicity test, drugs are tested at 5 or 6 logarithmically spaced dose levels against small groups of tumour-bearing animals. In choosing the dose range an attempt is made to exceed the toxic levels with the highest doses. An example of an experiment of this type in which the effects of TEM on the Walker tumour were tested is given in Table V.

Sugiura, under the sponsorship of the C.C.N.S.C. (Sugiura, 1963), has designed a much more comprehensive test (which may be likened to sea-fishing with a rather fine net). It involves using 31 different transplantable tumours of mouse, rat, hamster and chicken, including tumours of known viral origin. Over the past decade some 1000 compounds have been tested in this way. Clearly this number is a long way short of the 30,000 compounds per year with which the C.C.N.S.C. in 1959 were proposing to cope, and there has been a tendency for selected rather than unselected compounds to be fed into Suguira's comprehensive scheme. Thus what was perhaps intended as a primary test is in fact a secondary test.

A test (Section K.6 above) that is comprehensive in a different way is that of Garattini and Palma (1961). This is still less able to cope with large numbers of test compounds and cannot possibly be considered as a substitute for the C.C.N.S.C. primary test.

N. The Need for New Thinking and New Types of Test

Most of the clinically useful agents listed in Table VI came to light before large-scale testing of unselected compounds was organised.

TABLE IV

Routine screening procedures used by the Chester Beatty Research Institute, London

Transplantable tumour	Species	Strain	Evaluation of test agent
Walker carcinoma 256	Rat	C.B.	$\frac{C}{T} = \frac{\text{mean tumour weight for controls}}{\text{mean tumour weight for treated}}$
Established Walker carcinoma 256	Rat	C.B.	Reduction in tumour size measured with calipers
Osteogenic sarcoma D177	Rat	Wistar	$\frac{C}{T} = \frac{\text{mean tumour weight for controls}}{\text{mean tumour weight for treated}}$
August rat tumour	Rat	August	$\frac{C}{T} = \frac{\text{mean tumour weight for controls}}{\text{mean tumour weight for treated}}$
Adenocarcinoma 755	Mouse	(C57BL × DBA/2)F_1 Hybrid	$\frac{C}{T} = \frac{\text{mean tumour weight for controls}}{\text{mean tumour weight for treated}}$
Sarcoma 180	Mouse	Stock albino	$\frac{C}{T} = \frac{\text{mean tumour weight for controls}}{\text{mean tumour weight for treated}}$
3,4-Benzopyrene induced tumour	Rat	C.B.	Reduction in tumour size measured with calipers
Spontaneous mammary tumour	Mouse	C+	Reduction in tumour size measured with calipers
Furth leukaemia	Rat	Furth	Survival time of treated compared with control

TABLE V

The effects of logarithmically spaced doses of Thio-TEPA on the transplantable Walker carcinoma in rats: combined assessment of toxicity and anti-tumour activity

Solvent Water Date of implant 15/11/63 Date of 1st injection 16/11/63 No. of injections 1 Date killed 25/11/63

	Control	Dose 0·5 mg/kg		Dose 1·0 mg/kg		Dose 2·0 mg/kg		Dose 4·0 mg/kg		Dose 8·0 mg/kg		Dose 16·0 mg/kg	
	Tumour wt. (g)	Tumour wt. (g)	Body-wt. change (g)	Tumour wt. (g)	Body-wt. change (g)	Tumour wt. (g)	Body-wt. change (g)	Tumour wt. (g)	Body-wt. change (g)	Tumour wt. (g)	Body-wt. change (g)	Tumour wt. (g)	Body-wt. change (g)
	59												
	55												
	47	53	+35	8	+131	0	+103	0	+89	0	+30	Died 20/11/63	
	40	41	+54	3	+ 85	0	+ 94	0	+79	0	+60	Died 20/11/63	
	38	31	+86	0	+ 78	0	+ 78	0	+73	0	+49	Died 20/11/63	
	31												
Average tumour wt.	45	42		4		0		0		0			
% Tumour growth inhibition		7		91		100		100		100			
Average body-wt. change	+38		+58		+ 98		+ 92		+80		+46		

LD_{50} = 11·3 mg/kg; ED_{90} = 1 mg/kg; therapeutic index = LD_{50}/ED_{90} = 11·3.

1. The dose range chosen for the test was based on a preliminary estimation of toxicity, which gave the result

1 mg/kg—0/3 deaths 5 mg/kg—0/3 deaths 25 mg/kg—3/3 deaths 125 mg/kg—3/3 deaths

2. The average body-weight change is the difference between the mean body-weight at the beginning of the experiment and at the end of the experiment after dissection of the tumours. This change in body-weight is more sensitive than lethality as a measure of toxicity. A reduced rate of gain in weight is regularly seen in animals with rapidly growing tumours. This effect is seen in the control untreated animals and in those given the lowest dose of Thio-TEPA. At higher doses there is also less gain in body-weight, although the animals have no tumours; the adverse effect on body-weight is then attributable to the toxicity of the drug. With two compounds showing the same therapeutic index, the one causing less effect on the rate of gain in body-weight at high dose levels would be considered the more selective anti-tumour agent.

Since that time the new compounds that have come into clinical use have shown only moderate advantages over those previously available. Does this indicate that both the discriminating and the undiscriminating types of approach have failed?

The real hope of the C.C.N.S.C. indiscriminate test was that entirely new classes of active compound would be discovered empirically. To date this hope has not been realised. Some have argued that the

TABLE VI

Cancer chemotherapeutic agents in clinical use

Stilboestrol	Melphalan
Testosterone	Sarcolysin (≡ merphalan)
Prednisone	Chlorambucil
Cortisone	Mannitol mustard
Actinomycin D	Cytoxan
Mitomycin C	Nitrogen mustard (methyl di-2-chloroethylamine)
Vincaleukoblastine	Nitromin
Urethane	T.E.P.A. (triethylenephosphoramide)
Aminopterin	Thio T.E.P.A.
Amethopterin	T.E.M. (triethylenemelamine)
6-Mercaptopurine	E 39 (2,5-bis-(ethylene-imino)-3,6-dipropoxy-*p*-benzo-quinone)
	Myleran

requirement in terms of tumour inhibitory activity before a compound could pass the primary test, and so become a candidate for more careful examination, is too stringent. Others, including ourselves, think that too much emphasis has been put on antigrowth activity as a criterion of drug effect, and that compounds favourably affecting the course of the disease, in terms of invasion, metastasis and survival, may well have been overlooked (v. below).

In the United States there is at present in progress a serious reappraisal of that part of the C.C.N.S.C. programme dealing with the testing of unselected compounds. For years lip-service has been paid to the necessity for more basic research; much research that was called "basic" was in fact nothing of the sort. This seems to be the crux of the matters now under discussion. At the same time there is a likelihood that in future more emphasis will have to be given to the detection of carcinogenic hazards and to cancer prevention and less to the chemical approaches to cancer therapy. However, it is much more difficult to stop a programme than to start it. Consequently we may expect righteous indignation and ponderous justifications for several years to come. In the meantime it is necessary to make sure that the

best of our existing test methods and organisations are preserved until such time as truly fundamental research has improved our knowledge of the nature of the problem and we can use the tests on a more rational basis.

For discovery of new classes of compound we doubt whether there is any real substitute for careful observation and recording of biological phenomena in the course of all animal experimentation. The only way to force the pace, therefore, is to increase the amount of basic scientific work in this general area.

No drug that selectively destroys tumour cells while doing little or no damage to normal cells has yet been discovered. The rationale of this approach to the cancer problem was based on the assumption that the cancer cell could be likened to a microbial parasite and that, just as antibiotic drugs have been developed for dealing with the latter, so could chemotherapeutic agents be developed to deal with cancer cells. Ignoring much of what was known of the nature of cancer, microbiological ideas and techniques were borrowed in the development of testing techniques. From the beginning, the fact that tumour cells originate from normal cells, and that no two cancerous cells look or behave alike, rendered remote the chances of success for this approach. Now that it is clear that it has failed, it is an urgent problem to develop more profitable work for those engaged in the huge cancer chemotherapy testing programme. We have here described or referred to the numerous test methods developed during the last 15 years or so. The vast majority of them are for detecting the ability of agents to reduce the growth and reproduction of cancer cells. Yet the ability of the cancer cell to grow and divide may be the only feature it still has in common with the normal body cell from which it originated. It is the ability of the cancer cell to invade other tissues that distinguishes it from normal cells and endows it with lethal portent. Even the most rapid rate of tumour-cell multiplication does not exceed that of regenerating liver cells, gut mucosa cells or many other cells. If cancer cells could divide, but not invade, the great majority of tumours could be treated surgically. "Invasiveness" is then the property of cancer cells that (except for leukaemic cells, whose normal precursors can infiltrate other tissues) distinguishes them clearly from normal. This is the property we should attempt to attack by drugs or by other means. We should be trying to develop tests for inhibition of invasiveness. The basic research behind this approach has, however, hardly been begun. (Leighton, Kline, Belkin, Legallais and Orr, 1957; Ambrose and Easty, 1960; Ambrose and Wheatley, 1961; Ruhenstroth-Bauer, 1961a,b.) In the meantime, how can our existing approaches be adapted to the real problems of cancer instead of to the fanciful image of the disease as a manifestation of a microbiological infection?

First, we can take due cognisance of the fact that no two cancers are alike and that therefore we cannot hope to predict how particular tumours will respond to particular drugs unless we undertake special preliminary tests. Recent advances in tissue-culture techniques, whereby cells from a human cancer can be grown for several days in tissue culture, have made it possible to test the effect of several different anti-cancer agents on the tumour cells in vitro before therapy with any one of them is attempted. This type of test (discussed above in Section H), though still at an experimental stage, is rational and promising. However, it will not lead to the discovery of new drugs.

Secondly, we can study the biochemical nature of discrete varieties of human cancer to see which anti-cancer agents are likely to have the most beneficial anti-growth effect. At the same time we may be able to match particular types of human cancer with certain transplantable animal tumours so that the latter may be used as specific models for drug testing. Attempts to study the biochemical features of human cancer have begun in Wisconsin (Heidelberger, 1961) and in London (see Annotation in *British Medical Journal*, *i*, 1752, 1961).

A third approach involves the use of "minimum deviation tumours". In the past two years, as a result of the forceful arguments of V. R. Potter (1961), attention has turned to what have been termed "minimum deviation" tumours. Such tumours are conceived as consisting of cells differing from the normal cells from which they originated (by deletions) minimally in respect of their endowment of nucleoproteins, protein enzymes and metabolic capabilities. As a result of a search for tumours of this kind, the Morris Hepatoma 5123 was found (Morris, Sidransky, Wagner and Dyer, 1960; Potter, Pitot, Ono and Morris, 1960; and Pitot, 1960). At first it was thought that this slow-growing tumour differs little from normal liver cells in its biological, including biochemical, characteristics. With the passage of time, however, more and more differences have come to light. Morris (1963) has reviewed the potential value of this tumour for future studies.

Apart from these two approaches we can only go on trying, using existing test techniques to produce agents with a greater therapeutic ratio and fewer side effects than those we already have. The real hope of the future depends on the development of entirely new testing techniques.

Acknowledgments

We wish to record our sincere thanks to other members of the staff of the Institute of Cancer Research particularly concerned with the development and testing of chemotherapeutic agents, for advice and for providing us with illustrative examples. In this connection we are specially grateful to Professor A. Haddow, F.R.S., Professor F. Bergel, F.R.S., Dr. L. A. Elson, Dr. V. M. Rosenoer, Mr.

B. C. V. Mitchley and Mr. J. L. Everett. We also thank Mr. K. G. Moreman and Miss G. J. Coggin for valuable help with the illustrations and finally to Mrs. M. Walters, B.Sc. for reading, correcting and collating the manuscript.

The experimental work referred to in this chapter was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council (Tobacco Manufacturers' Benefaction), the British Empire Cancer Campaign, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

References

Abe, M., Miyaki, K., Mizuno, D., Narita, N., Takeuchi, T., Ukita, T. and Yamamoto, T. (1959). *Jap. J. Med. Sci. Biol.* **12**, 175.

Antikajian, G., Wright, L. T., Plummer, J. I. and Weintraub, S. (1952). *J. nat. Cancer Inst.* **12**, 269.

Ambrose, E. J., Andrews, R. D., Easty, D. M., Field, E. O. and Wylie, J. A. H. (1962). *Lancet. i*, 24.

Ambrose, E. J., Dudgeon, J. A., Easty, D. M. and Easty, G. C. (1961). *Exp. Cell Res.* **24**, 220.

Ambrose, E. J. and Easty, G. C. (1960). *Acta Un. Int. Cancr.* **16**, 36.

Ambrose, E. J. and Wheatley, D. N. (1961). *Rep. Brit. Emp. Cancer Campgn.* **39**, 117.

Aoki, T. and Fukuoka, F. (1959). *Gann.* **50**, 47.

Bahner, C. T. (1958). *Cancer Res.* **18**, Screening Data I, 537.

Baker, J. R. and Tregier, A. (1962). *Proc. Amer. Ass. Cancer Res.* **3**, 302.

Baserga, R., Putong, P. B., Tyler, S., and Wartman, W. B. (1960). *Brit. J. Cancer* **14**, 173.

Bather, R. (1960). *Acta. Un. Int. Cancr.* **16**, 545.

Bennette, J. G. (1960). *Nature, Lond.* **187**, 72.

Berenbaum, M. C. (1962). *Nature, Lond.* **196**, 384.

Bergel, F. (1960). *L.A.C.* Collected Papers, **9**, 39.

Bieber, S. and Hitchings, G. H. (1955). *Cancer Res. Suppl.* **3**, 80.

Biesele, J. J. (1954). *Ann. N.Y. Acad. Sci.* **58**, 1129.

Biesele, J. J. (1958). *Ann. N.Y. Acad. Sci.* **76**, 530.

Bittner, J. J. (1936). *Science* **84**, 162.

Blakemore, W. S., McKenna, J. M., McAllister, R. M. and Coriell, L. L. (1960). *Proc. Amer. Ass. Cancer Res.* **3**, 96.

Block, M. and Takano, G. (1952). *Cancer Res.* **12**, 250.

Boone, I. U., Strang, V. G. and Langham, W. H. (1958). *Cancer Res.* **18**, Screening Data I, 361.

Burchenal, J. M., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P. and Stock, C. C. (1949). *Cancer* **2**, 119.

Clarke, D. A. (1955). *Cancer Res. Suppl.* **3**, 14.

Clarke, D. A., Philips, F. S., Sternberg, S. S., Elion, G. B. and Hitchings, G. H. (1953). *Cancer Res.* **13**, 593.

Cloudman, A. M. (1941). *Science* **93**, 380.

Cobb, J. P. (1955). *Trans. N.Y. Acad. Sci.* II, **17**, 237.

Cobb, J. P. (1960). *Ann. N.Y. Acad. Sci.* 84, 513.

Cobb, J. P. and Walker, D. G. (1958). *J. nat. Cancer Inst.* **21**, 263.

Cobb, J. P., Walker, D. G. and Wright, J. C. (1961). *Cancer Res.* **21**, 583.

Creech, H. J., Breuninger, E., Hankwitz, R. F., Polsky, G. and Wilson, M. L. *Cancer Res.* **20**, Screening Data VII, 471.

Creech, H. J., Mauschka, T. S., Hankwitz, R. F., Littleton, B. J. and André, J. (1955). *Cancer Res. Suppl.* **3**, 47.
Curtis, A. S. G. (1960). *Med. biol. Illus.* **10**, 261.
Davidson, J. D. and Freeman, B. B. (1955). *Cancer Res. Suppl.* **3**, 97.
Dipaolo, J. A. (1962). *Fed. Proc.* **21**, 165.
Dixon, G. J., Schabel, F. M., Skipper, H. E., Dulmadge, E. A. and Duncan, B. (1961). *Cancer Res.* **21**, Screening Data XI, 535.
Dogg, C. P., Karnofsky, D. A., Stock, C. C., Lacon, C. R. and Roddy, J. (1955). *Proc. Soc. exp. Biol., N.Y.* **90**, 489.
Downing, V., Farber, S. and Abu-Hydar, M. (1953). *Proc. Amer. Ass. Cancer Res.* **1**, 13.
Druckrey, H. (1959). *Acta Un. Int. Cancr.* **15** (Suppl.), 85.
Eagle, H. (1958). *Ann. N.Y. Acad. Sci.* **76**, 496.
Eagle, H. and Foley, G. E. (1956). *Amer. J. Med.* **21**, 739.
Eagle, H. and Foley, G. E. (1958). *Cancer Res.* **18**, 1017.
Easty, D. M., Wylie, J. A. H. and Yarnell, M. M. (1961). *Rep. Brit. Emp. Cancer Campgn* **39**, 132.
Eddy, B. E., Grubbs, J. E. and Young, R. D. (1962). *Proc. Soc. exp. Biol., N.Y.* **111**, 718.
Fortner, J. J. and Gale, A. (1958). *Proc. Amer. Ass. Cancer Res.* **2**, 297.
Foulds, L. (1954). *Cancer Res.* **14**, 327.
French, F. A. and Freedlander, B. L. (1960). *Cancer Res.* **20**, Screening Data VII, 505.
Friend, C. (1957). *J. exp. Med.* **105**, 307.
Fujikawa, M. (1959). *Folia Pharmacol. Japon.* **55**, 1442.
Furst, A., Cutting, W. and Gross, H. S. (1958). *Cancer Res.* **18**, Screening Data I, 417.
Gallily, R. and Woolley, G. W. (1958). *Ann. N.Y. Acad. Sci.* **76**, 791.
Gardner, W. U., Dougherty, T. F. and Williams, W. L. (1944). *Cancer Res.* **4**, 73.
Garattini, S. and Palma, V. (1961). *In* "Biological Approaches to Cancer Chemotherapy" (ed. R. J. C. Harris), p. 167. Academic Press, London.
Gellhorn, A., Engelman, M., Shapiro, D., Graff, S. and Gillespie, H. (1950). *Cancer Res.* **10**, 170.
Gellhorn, A. and Hirschberg, E. (1955). *Cancer Res. Suppl.* **2**, 13.
Gellhorn, A., Kells, A. and Golino, M. (1955). *Cancer Res. Suppl.* **3**, 38.
Gellhorn, A., Peterson, E. R., Kells, A., Hirschberg, E. and Murray, M. R. (1954–55). *Ann. N.Y. Acad. Sci.* **60**, 273.
Goldacre, R. J. and Sylven, B. (1959). *Nature, Lond.* **184**, 63.
Goldin, A., Venditti, J. M. and Kline, I. (1962). *Cancer Res.* **22**, Screening Data XIV, 157.
Gottfried, B., Padnos, M. and Molomut, N. (1963). *Nature, Lond.* **197**, 1309.
Griffiths, J. D., Hoppe, E. and Cole, W. H. (1961). *Cancer* **14**, 111.
Haddow, A., Harris, R. J. C., Kon, G. A. R. and Roe, E. M. F. (1948). *Trans. roy. Soc.* **A241**, 147.
Harding, H. E. and Passey, R. D. (1930). *J. Path. Bact.* **33**, 417.
Handler, A. H. (1958). *Ann. N.Y. Acad. Sci.* **76**, 775.
Handler, A. H., Patt, D. I. and Lutz, B. R. (1952). *Anat. Rec.* **112**, 449.
Harris, J. J. (1958). *Ann. N.Y. Acad. Sci.* **76**, 764.
Harris, J. J. (1962). *Cancer Res.* **22**, Screening Data XIII, 1.
Hayes, D. M., Spurr, C. L. and Hines, J. D. (1962). *Cancer Chemotherapy Rep.* **24**, 1.

Heidelberger, C. (1961). *Nature, Lond.* **189**, 627.
Heidelberger, C. and Baumann, M. E. (1958). *Cancer Res.* **18**, Screening Data I, 373.
Heidelberger, C. and Keller, R. A. (1955). *Cancer Res., Suppl.* **3**, 106.
Hemmerly, J. and Demerec, M. (1955). *Cancer Res., Suppl.* **3**, 69.
Henderson, J. F. and Junga, I. G. (1961). *Cancer Res.* **21**, Screening Data IX, 7.
Hirschberg, E. (1958). *Cancer Res.* **18**, 869.
Hirschberg, E. and Merson, G. (1955). *Cancer Res., Suppl.* **3**, 76.
Hitchings, G. K., Elion, G. B., Falo, E. A., Russell, B. P., Sherwood, M. B. and Vanderwerff, K. (1950). *J. biol. Chem.* **183**, 1.
Holland, J. F., Guthrie, R., Tieckelmann, H. and Cuddiny, R. (1958). *Cancer Res.* **18**, Screening Data I, 335.
Hoshi, T. and Hata, T. (1958). *J. Antibiotics* Ser. A. **11**, 99.
Huebner, R. J. (1959). *In* "Perspectives in Virology" (ed. M. Pollard), Vol. I, p. 121. Wiley, New York.
Imanaga, H., Kondo, T., Maruyana, T., Tsukui, K. and Nakazato, H. (1959). *Acta Un. Int. Cancr.* **15** (Suppl.), 134.
Jaenner, H. and Krumme, H. (1960). *Arzneimittelforsch.* **10**, 563.
Karnofsky, D. A. (1955). *Cancer Res.*, Suppl. **3**, 83.
Karnofsky, D. A. and Lacon, C. R. (1962). *Cancer Res., Suppl.* **22**, Cancer Chemotherapy Screening Data XIII, 84.
Kidder, G. W. and Kewey, V. C. (1949). *J. biol. Chem.* **179**, 181.
Klein, G. (1951). *Exp. Cell Res.* **2**, 518.
Koller, P. C. (1960). *In* "Cell Physiology of Neoplasia". University of Texas Press, Austin.
Kondo, T. and Moore, G. E. (1961). *Cancer Res.* **21**, 1396.
Larionov, L. F. (1962). "Chemotherapy of Experimental Tumours". Medgiz, Moscow.
Lasagna, L. (1958). *Ann. N.Y. Acad. Sci.* **76**, 939.
Law, L. W., Dunn, T. B., Boyle, P. J. and Miller, J. H. (1949–50). *J. nat. Cancer Inst.* **10**, 179.
Leighton, J., Kline, I., Belkin, M., Legallais, F. and Orr, H. C. (1957). *Cancer Res.* **17**, 359.
Leiter, J., Wodinsky, I., Bourke, A. R. and Schneiderman, M. A. (1959). *Cancer Res.* **19**, Screening Data IV, 488.
Le Page, G. A. and Greenlees, J. L. (1955). *Cancer Res. Suppl.* **3**, 102.
Lettre, H. (1950). *Z. Krebsforsch.* **57**, 1.
Loewenthal, H. and Jahn, G. (1932). *Z. Krebsforsch.* **37**, 439.
Luck, J. M. (1956). *Science* **123**, 984.
Luck, J. M. (1957). *Cancer Res.* **17**, 1071.
Lutz, B. R., Fulton, G. P., Patt, D. I., Handler, A. H. and Stevens, D. F. (1951). *Cancer Res.* **11**, 64.
Marsh, W. S. and Cullen, M. R. (1958). *Ann. N.Y. Acad. Sci.* **76**, 752.
Matthies, E. (1961). *Cancer Res.* **21**, Screening Data IX, 1.
McAllister, R., Grunmeier, P. W., Coriell, L. L. and Blakemore, W. S. (1959). *Cancer.* **12**, 938.
Merchant, D. J. and Eidam, C. R. (1961). *Advanc. appl. Microbiol.* **3**, 109.
Merker, P. C., Anido, P., Sarino, J. and Woolley, G. W. (1962). *Cancer Res., Suppl.* **22**, Cancer Chemotherapy Screening Data XIII, 9.
Merker, P. C., Baba, T. and Singer, K. (1960). *Cancer Res.* **20**, 1462.
Merker, P. C., Kimobo, H., Pearle, F. K. and Anido, R. (1961). *Proc. Amer. Ass. Cancer Res.* **3**, 250.
Minster, J. (1962). *Proc. Amer. Ass. Cancer Res.* **3**, 344.

Miyamura, S. (1956). *Antibiotics and Chemotherapy* 6, 1280.
Moore, A. E. (1962). *Virology* **18**, 182.
Morris, H. P. (1963). *In* "Progress in Tumour Research" (ed. F. Homburger), p. 370. S. Karger, Basel and New York.
Morris, H. P., Sidransky, H., Wagner, B. P. and Dyer, H. M. (1960). *Cancer Res.* **20**, 1252.
Murphy, J. B. (1912). *J. Amer. med. Ass.* **59**, 874.
Murphy, J. P. F., Zipf, R. E., Parrish, W. B. and Katchman, B. J. (1961). *Clin. Chem.* **7**, 568.
Nakahara, W. (1922). *J. exp. Med.* **35**, 493.
Nakahara, W. (1924). *J. exp. Med.* **40**, 363.
Nakahara, W. (1925). *J. exp. Med.* **41**, 342.
Navashiu, S. M., Fomina, I. P. and Terent'eva, T. G. (1960). *Antibiotiki* **5**, 53.
Oettel, H. and Wilhelm, G. (1958). *Cancer Res.* **18**, Screening Data I, 425.
Okubo, S., Ikeda, K., Yamada, I. and Sugawara, H. (1951). *Gann.* **42**, 268.
Old, L. J., Benacerraf, B., Clarke, D. A., Carswell, E. A. and Stockert, E. (1961). *Cancer Res.* **21**, 1281.
Osato, S., Oda, K., Toshida, K. and Takahashi, S. (1951). *Gann.* **42**, 269.
Owens, A. H. and Busch, G. J. (1961). *Fed. Proc.* **20**, 157.
Owen, L. N. (1960). *Nature, Lond.* **187**, 795.
Oyama, V. I. and Eagle, H. (1956). *Proc. Soc. exp. Biol., N.Y.* **91**, 305
Palm, J. E., Teller, M. N., Merker, P. C. and Wooley, G. W. (1958). *Ann. N.Y. Acad. Sci.* **76**, 812.
Patt, D. I., Handler, A. H. and Lutz, B. R. (1951). *Anat. Rec.* **111**, 521.
Patt, H. M. and Straube, R. L. (1955–56). *Ann. N.Y. Acad. Sci.* **63**, 728.
Pienta, R. J., Bernstein, E. H. and Groupé, V. (1961). *Fed. Proc.* **20**, 439.
Pitot, H. C. (1960). *Cancer Res.* **20**, 1262.
Potter, M. and Boyce, C. R. (1962). *Nature, Lond.* **193**, 1086.
Potter, M. and Fahey, J. L. (1960). *J. nat. Cancer Inst.* **24**, 1153.
Potter, M., Fahey, J. L. and Pilgrim, H. I. (1957). *Proc. Soc. exp. Biol., N.Y.* **94**, 327.
Potter, M. and Robertson, C. L. (1960). *J. nat. Cancer Inst.* **25**, 847.
Potter, V. R. (1961). *Cancer Res.* **21**, 1331.
Potter, V. R., Pitot, H. C., Ono, T. and Morris, H. P. (1960). *Cancer Res.* **20**, 1255.
Reilly, H. C., Stock, C. C., Buckley, S. M. and Clarke, D. A. (1953). *Cancer Res.* **13**, 684.
Riley, V. (1959). *Proc. Soc. exp. Biol., N.Y.* **100**, 155.
Riley, V. and Wroblewski, F. (1959). *Fed. Proc.* **18**, 310.
Rous, P. (1911). *J. Amer. med. Ass.* **56**, 198.
Ruhenstroth-Bauer, G., Kübler, G., Fuhrmann, G. F. and Rueff, F. (1961a). *Klin. Wschr.* **39**, 764.
Ruhenstroth-Bauer, G., Straub, E., Sachtleben, P. and Fuhrmann, G. F. (1961b). *Münchener Med. Wschr.* **103**, 794.
Sartorelli, A. C., Kruse, P. F., Booth, B. A. and Schoolar, E. J. (1960). *Cancer Res.* **20**, Screening Data VII, 495.
Sassenrath, E. N., Costa, F. M. and Greenberg, D. M. (1958). *Cancer Res.* **18**, Screening Data I, 341.
Sassenrath, E. N., Costa, F. M. and Greenberg, D. M. (1961). *Cancer Chemotherapy Rep.* **11**, 5 and 13.
Sato, H. (1959). *Acta path. Jap.* **9** (Suppl.), 685.
Sato, H. (1961). *Cancer Chemotherapy Rep.* **13**, 33.

Schabel, F. M., Montgomery, J. A., Skipper, H. E., Lastar, W. R. and Thomson, J. R. (1961). *Cancer Res.* **21**, 690.
Schatten, W. E. (1962). *Cancer Res.* **22**, 286.
Schepartz, S. A., Wodinsky, I. and Leiter, J. (1962). *Cancer Chemotherapy Rep.* **19**, 1.
Scholler, J. and Bittner, J. J. (1958). *Cancer Res.* **18**, 464.
Scholler, J., Bittner, J. J. and Philips, F. S. (1957). *Cancer Res.* **17**, 605.
Scholler, J., Philips, F. S., Sternberg, S. S. and Bittner, J. J. (1956). *Cancer* **9**, 240.
Schrek, R. (1935). *Amer. J. Cancer.* **24**, 807.
Schrek, R. (1936a). *Amer. J. Cancer.* **28**, 345.
Schrek, R. (1936b). *Amer. J. Cancer.* **28**, 364.
Schultz, J., Rothman, N. and Aronson, M. M. (1955). *Cancer Res.*, Suppl. **3**, 86.
Schuurman, D., Duncan, D. T. and Olson, R. M. (1960). *Antibiotics and Chemotherapy* **10**, 535.
Shabad, L. M., Loginov, A. V. and Volfson, N. I. (1959). *Trudy Simpoziuma Ann.*, Moscow, Medgiz, 1955; see *Cancer Chemotherapy Abstr.* **2**, 4105 (1961).
Stock, C. C. (1960). *Nat. Cancer Inst. Monograph.* No. 3, p. 23.
Stock, C. C., Clarke, D. A., Philips, F. S. and Barclay, R. K. (1958). *Cancer Res.* **18**, Screening Data I, 49.
Sugiura, K. (1953). *Cancer Res.* **13**, 431.
Sugiura, K. (1955). *Cancer Res., Suppl.* **3**, 18.
Sugiura, K. (1959). *Gann.* **50**, 251.
Sugiura, K. (1961). *In* "Progress in Experimental Tumour Research" (ed. F. Homburger), Vol. 2, p. 334. S. Karger, New York.
Sugiura, K. (1962a). *Cancer Res.* **22**, Screening Data XIII, 93.
Sugiura, K. (1962b). *Cancer Chemotherapy Rep.* **22**, 41.
Sugiura, K. and Creech, H. J. (1955–56). *Ann. N.Y. Acad. Sci.* **63**, 962.
Sugiura, K. and Stock, C. C. (1952). *Cancer.* **5**, 382.
Takayama, S. and Woolley, G. W. (1958). *Ann. N.Y. Acad. Sci.* **76**, 797.
Tannenbaum, A. and Silverstone, H. (1953). *Advanc. Cancer Res.* **1**, 452.
Tarnowski, G. S., Bane, H. N., Conrad, J., Nickson, J. J., Stock, C. C. and Sugiura, K. (1958). *Cancer Res.* **18**, Screening Data I, 225.
Tarnowski, G. S. and Bates, L. A. (1961). *Cancer Res.* **21**, Screening Data XI, 377.
Teller, M. N. (1962). *Cancer Res.* **22**, Part II, 25.
Teller, M. N., Merker, P. C., Palm, J. E. and Woolley, G. W. (1958a). *Ann. N.Y. Acad. Sci.* **76**, 742.
Teller, M. N., Palm, J. E., Merker, P. C., Harris, J. J. and Woolley, G. W. (1958b). *Cancer Res.* **18**, 522.
Teller, M. N., Merker, P. C., Palm, J. E. and Woolley, G. W. (1960). *Cancer Res.* **20**, 112.
Tiunov, L. A. (1960). *Vop. Onkol.* **6**, 37; see also *Cancer Chemotherapy Abstr.* **1**, 1716 (1960).
Toolan, H. W. (1953). *Cancer Res.* **13**, 389.
Toolan, H. W. (1954). *Cancer Res.* **14**, 660.
Toolan, H. W. (1958). *Ann. N.Y. Acad. Sci.* **76**, 733.
Toplin, I. (1959). *Cancer Res.* **19**, 959.
Valyi-Nagy, T., Hernady, F., Szabo, C. and Jeney, A. (1961). *Antibiotics and Chemotherapy* **11**, 238.
Vedrich, M., Greene, O. M. and Greenberg, J. (1961). *Cancer Res. Suppl.* **21**, Cancer Chemotherapy Screening Data XI, 359.
Walpole, A. L. (1951). *Brit. J. Pharmacol.* **6**, 135.

Weiant, E. A., Wodinsky, I., Kensler, C. J. and Leiter, J. (1961). *Proc. Amer. Ass. Cancer Res.* **3**, 276.

Wodinsky, I., Kensler, C. J., Leiter, J., Little, A. D. and Weiant, J. (1961). *Proc. Amer. Ass. Cancer Res.* **3**, 276.

Woodhouse, D. L. (1947). *Cancer Res.* **7**, 398.

Woolley, G. W. (1953). *Cancer Res.* **13**, 327.

Woolley, G. W. (1962). *Cancer Res.* **22**, Screening Data XIII, 34.

Wright, J. C., Cobb, J. P., Gumport, S. L., Golomb, F. K. and Safadi, D. (1957). *New England. J.* **257**, 1207.

Wright, J. C., Cobb, J. P., Gumport, S. L., Safadi, D., Walker, D. G. and Golomb, F. M. (1962). *Cancer* **15**, 284.

Yamamoto, T., Nishioka, K., Osa, A., Sakurai, T., Yoshida, T. and Kikugawa, H. (1959). *Acta Un. Int. Cancr.* **15**, 306 (in *Cancer Chemotherapy* Supplement).

Yoshida, T. (1949). *Gann.* **40**, 1.

Yoshida, T., Sato, H., Nagasawa, B., Atsumi, A., Kobayashi, K., Hosokawa, K., Nakamura, K., Ishidate, N. and Sakurai, Y. (1950). *Gann.* **41**, 93.

Yushok, W. D. (1958). *Cancer Res.* **18**, Cancer Chemotherapy Screening Data, 1, 379.

Zimmerman, H. M. and Arnold, H. (1941). *Cancer Res.* **1**, 919.