EXPERIMENTS IN CARCINOGENESIS, 1939 - 1944.

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In submitting this thesis for the degree of Doctor of Medicine, the following five papers and their commentaries are presented as a series of experiments in carcinogenesis carried out, under war-time conditions, in the Wilkie Surgical Research Laboratory, University of Edinburgh. They record the evolution of an outlook rather than the solution of a problem; though in the course of these investigations, several points of material interest to the cancer worker have been clarified. Finally, the problem is summed up in general terms and a bridgehead for further work is established.

M. D., 1945.

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"An hypothesis which becomes dispossessed by new facts, dies an honourable death; and if it has already called up for examination those truths by which it was annihilated, it deserves a monument of gratitude."

(Henle, 1850).

"My experience teaches me that it is better to hold a well-understood and intelligible opinion, even if it should turn out to be wrong, than to be content with a muddle-headed mixture of conflicting views, sometimes miscalled impartiality, and often no better than no opinion at all."

(Bayliss, 1924).

"Yet since what one thinks determines what one does in cancer research, as in all else, it is well to think something. And it may prove worth while to think that one or more tumours of unknown cause are due to viruses."

(Peyton Rous, 1943).

INTRODUCTION.

The very success which has crowned so many aspects of scientific investigation tends to obscure the fact that when, some 300 years ago, man first turned from philosophy to experiment, there was no 'a priori' reason to suppose - still less, a guarantee - that the search for order by experimental means would be successful. Yet within a century - and particularly in the realms of physics, chemistry and natural science - the new scientific attitude gained some of its most striking achievements. The sequence of work and thought, for example, from Mendeléeff through Faraday and Clark Maxwell to the Curies and the new philosophy of Einstein, presents an almost unbroken chain of scientific triumphs, the full implications of which have yet to be realised. In like manner, Mendel and Darwin pointed the way to order in the vegetable and animal kingdoms of the early philosophers; and, as in the case of physics and chemistry, brought unitarian principles to bear in their respective fields. In medicine, on the contrary, empiricism and the dead hand of the Greeks for long held any serious advance in check. True, there were men of the calibre of Harvey and John Hunter - both masters of the experimental method; still later, the discoveries of anaesthesia and antisepsis opened, to surgeons in particular, wide new fields for the exploitation of their craft. Nevertheless, it is only within the present century that medicine as a whole has availed itself, in the conquest of disease, both of the attitude and the discoveries of contemporary science. Even the pioneer work of Paul Ehrlich was largely of an empirical nature, though Ehrlich was undoubtedly inspired by an outlook far in advance of his time.

Yet each new question which is posed and each new problem which is chosen for attack, labour under exactly those overriding and limiting factors which faced the experimental method at its inception. Now, as then, two questions are relevant. First, is the problem well founded? Second, is it likely that the particular problem can be resolved in the known terms of laboratory methods?

That the problem of cancer is well founded - that malignant disease is a biological entity - is now widely accepted. That the riddle/

/riddle of cancer can be solved in the laboratory has yet to be proved. For of all the medical problems of the present day which remain unsolved, the cancer problem supplies perhaps the most striking example. During the past 40 years the question of the aetiology of cancer has withstood the most corrosive reasoning and the most exacting experiment. One of the greatest tributes which can be paid to the genius of Paul Ehrlich lies in his rejection of the cancer problem as a study in research during his own time by his realisation that contemporary methods were not sufficiently advanced to make success probable. Seeking an alternative subject for his talents, Ehrlich chose syphilis - with the success which history records.

In entering the cancer field, then, the investigator may well repeat the second of Ehrlich's questions - "Is it likely that the problem can be solved in terms of current techniques?" If not, one may add, "In what direction must we look for further advances?"

Paper I. "Sulphydryl compounds and wound repair." (1940).

Even at the present time, experimental cancer research is still restricted by the fact that any study of abnormal and autonomous growth requires comparison with normal growth processes before valid deductions can be made. Indeed, it is possible that the problem of cancer may ultimately be stated as a 'reductio ad absurdum' in that, the factors which control normal growth being fully understood, malignant tissue will be found merely to be deficient in one such respect. Yet, at the time of writing, surprisingly little is known of the physical and chemical changes which take place in those cells which are capable of further division when their phase of growth is again replaced by a state of fixity, differentiation and function. Neither the workers in tissue culture on the one hand, nor the experimental embryologists on the other, have as yet selectively localised either the 'formative stimulus' to growth or the exact nature of the 'organiser substance' which, in the embryo at least, turns growth to differentiation and thereby preserves the integrity of the organism as a single functioning unit.

With a view to obtaining more insight into the processes of normal cell division, preliminary experiments were undertaken before the War to enquire further into the suggestions of Hammett and his school that the sulphydryl (syn: -SH, thiol, mercaptan) radicle in the cell nucleus is specifically concerned with the process of mitotic division. The inhibiting effects of high dilutions of arsenic and mercury salts on simple explants of chick embryo fibroblasts in Tyrode solution were confirmed; and the suggestion of Ephrussi was noted that the almost catalytic effect of embryo extract as a growth stimulant 'in vitro' is dependent upon the presence in the extract of a positive nitoprusside reaction for sulphydryl groups. Several compounds containing labile - SH groups were tested in wounds in man and animals; though it was realised that the dramatic improvement in epithelisation which was often observed was, in large wounds at least, not usually sustained. In addition to sulphydryl, other and as yet unknown limiting factors must be at work. Nevertheless, while rejecting the sweeping assertion of Hammett that "sulphydryl is the essential stimulus to growth by increase in cell number", the fact that organic sulphur <u>is</u> concerned in the process/

/process of mitotic division, places emphasis on the probable site of control of division in normal cells (and, 'ipso facto', the probable site of failure to control growth in cancer cells) namely, the proteins of the cell nucleus. Reference will again be made to this point in the summary at the end of this thesis.

With the advent of the War, further laboratory investigation on this particular subject had to be withheld. "Sulphydryl compounds and wound repair" was written as a preliminary report in Orkney during the 'Battle of Britain' and at a time when the possibility of further academic work seemed remote indeed. Later, and as a result of this paper, Fearon, in Dublin, synthesised thiourea and tested it on wounds, and made the suggestion in a personal communication that the -SH group might be important in wound healing by protecting aseorbic acid in its rôle of collagen former.

In the cancer field, several interesting papers have subsequently appeared. Crabtree, in London, has found that substances such as organic chlor-compounds, bromo-benzene and maleic anhydride (all of which have the common property of fixing labile sulphydryl groups) will inhibit the dermal action of the highly potent carcinogen, benzpyrene. Fieser, in America, has suggested from chemical considerations that the point of attack of the carcinogenic hydrocarbons on the living cell may reside in the disulphide linkages of the cell proteins. In this respect, it is of interest that a synthetic mercapturic acid derivative of benzpyrene is inactive in inducing tumours when applied repeatedly to the skin of mice.

In an unpublished experiment, I have confirmed the inhibitory action of bromo-benzene when applied alternately with benzpyrene to the skin of mice, and have excluded the possibility that the action is due to increased photosensitisation of the benzpyrene by the halogen. Tumours appeared almost at the same time and rate in mice painted alternately with bromo-benzene and benzpyrene in red light and kept in the dark, as in a group of animals similarly painted and exposed to sunlight. Nor did a third group of animals in which haematoporphyrin was injected during the period of surface applications of benzpyrene differ in regard to tumour emergence from the controls which received benzpyrene alone.

It/

/It is probable that much more will be heard of sulphydryl groups in normal and cancer cells during the next few years; and in this respect, the work of Rhoads and his group at the Memorial Hospital, New York, on the action of the split-products of the azo-dyes in liver cancer, is worthy of careful scrutiny. Here, too, the carcinogen appears to act specifically on the protein component of an enzyme system which is subject to thiol regulation - i.e: the important energy-yielding oxido-reduction of phosphorylating carbohydrate breakdown. Working with the hydrocarbons themselves, Potter has claimed a similar inhibitory effect in the thiol-regulated enzyme-system, succinoxidase, by which electron transfer with the cytochromes is brought about.

PAPER I.

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SULPHYDRYL COMPOUNDS AND WOUND REPAIR

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Wounds, their causation, pathology, and management, are at the present time subjects of more than usual importance. Even in peacetime almost every hospital shelters patients —victims of trauma, burning, sepsis, or venous stasis— in whom epithelization is virtually at a standstill and in whom only the end-results of scarring and deformity can be anticipated. The economic importance of hastening healing, both to patient and to hospital, need hardly be emphasized.

In this paper an attempt is made to summarize our knowledge of the broad principles underlying wound healing (and especially of epithelization), with particular attention to the part played in this process by the sulphydryl (–SH) radicle. Some of my preliminary work will be mentioned in the hope that it may form a basis for further experiment and research.

Broad Principles of Wound Repair

Of the four main types of tissue of which the animal organism is composed—nerve, muscle, connective tissue, and epithelium—only two, connective tissue and epithelium, show, in the mammalia at least, any marked powers of regeneration or repair. This they do by virtue of the fact that, given conditions where proliferation is required, such a cell can change from its normally fixed and differentiated form into one at the same time more simple and more mobile. Thus the elongated fibroblast can become similar to and perhaps identical with the macrophage; the surface epithelial cell ceases to form keratin and becomes

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amoeboid. "The epithelia of the mucous membranes in the course of repair pass through stages similar to those just described in the case of the epidermis. Thus the columnar cells, ciliated cells, or squamous cells all become rounded, cilia are lost, and the cells become mobile. As repair proceeds the cells regain the characters of the mother-cells from which they originally developed. . . . Thus in the case of columnar ciliated epithelium the cells which go to form the new layer, from being spherical and mobile, become first fixed, then gradually columnar, and finally ciliated" (Gray, 1937).

Here, then, is the first broad principle of wound repair —the power in certain cells of temporary "modulation" from a fixed to a mobile form, so that actual transport of the cell from place to place can be effected.

An important difference exists, however, in the healing of small and large wounds. In the *small* wound, migration, both of epithelium and of underlying connective tissue, is all that is required; mitosis, if any, occurs after healing is complete, and then only as a compensatory mechanism around the fringes of the wound. In the *large* wound, however, whether caused primarily or by the breaking down by sepsis of the original wound, migration alone is insufficient. First the wound becomes filled with a temporary scaffolding of "granulation tissue" and, in the case of the covering epithelium, migration has to be eked out by cell division (Arey, 1932). The larger the wound the more important does growth by cell division become; and it is in such wounds that we are interested. In passing we may note that granulation tissue is merely a series of mushroom-like masses composed of blood vessels and young connective tissue, and that the longer this temporary tissue is needed before the covering epithelium is laid down the more gross will be the ultimate scarring as organization among the fibroblasts takes place.

The second principle, therefore, is that in small wounds cell migration alone can effect healing. In large wounds cell division is also required: the larger the wound the more important does this factor of growth by cell division become.

Mutual Interaction of Connective Tissue and Epithelium

We may next perhaps ask ourselves: "What, if any, are the mutual effects on one another of these two cell types—the epithelial and the mesenchymal?" The answer is still in dispute.

In tissue culture it is well known that both fibroblasts and epithelium grow readily in pure culture, migration and mitosis being often observed. On adding fibroblasts to a culture of epithelium, however, Drew (1923) noted what he called an "organizing effect" by the fibroblasts on the epithelium, the epithelial cells once more becoming differentiated and fixed.

Turning to the mutual interaction of these cells in tissue fragments, F. H. Bentley (1936), on the other hand, observing an upsweeping of fibroblasts towards an overlying edge of proliferating epithelium at the margin of a wound, regarded this as a possible organizing effect, not by the fibrous tissue on the epithelium but by the epithelium on the underlying fibrous tissue. An extract prepared from the epithelium, however, failed to reproduce the characteristic pattern in the fibroblastic substrate. The converse experiment was not attempted. More recently Medawar (1938) has been able to prepare an extract (probably an aldehyde) which is a specific, reversible, and non-toxic inhibitor of mesenchyme. This when added to a mixed culture gives rise to a marked increase in the growth of the epithelium as the activity of the fibroblasts becomes depressed.

Though the evidence on both sides is copious and conflicting it would appear probable that the growth of epithelium is opposed by that of adjacent fibroblasts, and that the depression of excessive fibroblastic activity would assist the more rapid epithelization of a wound.

The Essential Stimulus to Proliferation

It was Alexis Carrel (1924) who pointed out that the stimulus to proliferation in cells appeared to lie in the presence of dead and dying tissue around them, and, later, that embryo extract and the extract of leucocytes were particularly powerful in this respect. Tissue culture has perhaps over-emphasized this aspect of cellular life, for it is only by delayed-culture methods that the second phase—namely, differentiation—can be studied. There are, moreover, at least two distinct aspects of the undifferentiated phase which should be, but seldom are, regarded separately: first, the stage of amoeboid movement; and, secondly, growth by mitotic division. Cells grown in embryo extract exhibit both phenomena.

It was natural that the properties of embryo extract should soon be tested *in vivo* (Carnot and Terris, 1926; Nakumara, 1930), and I have personally confirmed the striking healing properties of chick-embryo extract in

this respect. Unfortunately, however, embryo extract is not particularly easy to prepare and quickly loses its growth-promoting powers, so that from the therapeutic point of view it is impracticable. An attempt was made, therefore, to narrow down the actual formative stimulus, the first stage being its localization by Carrel and Baker (1926) in the higher split products of proteins. At a later date Baker (1929) found the presence of glutathione and haemoglobin together to have growth-promoting powers on fibroblasts *in vitro*.

It was in 1929, however, that Hammett (1929a, 1929b) and Hammett and Reimann (1929) first published their theory that growth by increase in cell numbers—i.e., by mitotic division—was dependent upon the presence of the sulphydryl (–SH) radicle; and, further, that repair of a wound by mitotic division depended on the disturbed balance between reduced sulphur (–SH) and oxidized sulphur (S–S) being restored. A somewhat similar view was expressed by Rapkine (1930). In this view, therefore, wound repair represents a mass action between sulphydryl (–SH) groups unmasked by the denaturation of the proteins from the injured tissues and the sulphoxide (S–S) groups into which they become rapidly converted; for –SH groups are themselves unstable and quickly break down. Further, as in any mass action, the products of the reaction—i.e., S–S groups—are themselves responsible for bringing the reaction to a standstill. Reduced to the simplest terms, therefore, –SH groups determine growth by mitosis: S–S groups, on the contrary, are the marks of differentiation. Again, it must be emphasized that the other important factor of growth—amoeboid movement—has not been mentioned.

Relation of Cell Stimulation and Cell Inhibition to the Genesis of Malignancy

If we are, at will, to bring about the abnormal behaviour of cells in the shape of acceleration and retardation of mitosis we must first exclude the possibility of unwanted side actions from these procedures.

Since malignant cells grow quickly, it has often been assumed that the stimulation of cells to a mitosis rate greater than normal might in itself have some bearing on the genesis of tumours. On close analysis, however, my own view is in accordance with that of Haddow (1938), who stated that "the local conditions which lead to tumour emergence are *not* such as primarily favour growth, but rather the reverse." In other words, the

known carcinogenic agents (for example, small doses of *x* rays over long periods ; the carcinogenic hydrocarbons) are inhibitors of growth rather than anything else.

With this in mind I painted a number of mice with one of the hydrocarbons for a period of time short of producing tumours, and later placed wounds of a standard size in the areas of depilation. These wounds were therefore under the worst physiological conditions as regards tumour emergence. One-third of the animals were kept as controls, one-third of the wounds were treated with fresh embryo extract, and the rest of the wounds were dressed with heparin (as used in delayed-culture methods *in vitro*). Although one or two tumours arose in the controls, by far the greater number appeared in the heparinized—i.e., inhibited—wounds, and none at all in those treated with embryo extract. The latter, of course, healed the most rapidly. No wide conclusions can be drawn from such an experiment, but it is interesting to note that a similar anti-carcinogenic action when using a synthetic sulphydryl compound together with one of the carcinogenic hydrocarbons has been recorded in America by Reimann and Hall (1936). In parenthesis, it may perhaps be remarked that although cells have been kept in tissue culture at an accelerated rate of mitosis for upwards of twenty years there has been no sign of a change in the direction of malignancy as tested by subsequent reimplantation of the cells into a suitable "host."

In the absence of wide evidence to the contrary, therefore, one feels that the physiological stimulation of cells to divide has no bearing in causing tumours and may possibly have the reverse effect. The deliberate inhibition of growth for long periods of time, on the other hand, should in the present state of our knowledge be treated with caution.

Clinical Aspects of Sulphydryl Therapy

It was naturally but a brief step from the identification of the sulphydryl radicle and its association with mitotic division to its clinical application in the actual treatment of wounds.

Many compounds contain the –SH radicle, but not all are suitable for therapy. Glutathione (Brunsting and Simonsen, 1933), for example, though active, is ruled out on the grounds of expense alone. In this connexion, however, it may be noted that glutathione has been successfully used in the reactivation of tissue-culture media after mitosis had ceased (Verne and Verne-Soubiran, 1938).

Following preliminary experiments in animals (Hammett and Reimann, 1929) and in man (Reimann and Hammett, 1929), Reimann (1930) obtained good results in a variety of wounds using a 1 in 10,000 solution of *p*-thiocresol as an intermittent dressing. Thioglucose, used previously, appeared to favour bacterial as well as tissue growth, and thiophenol tended to be too irritating. Thiocresol was chosen so that the cresol element might split off and act as an antiseptic. An ointment of approximately 0.5% thiocresol in lanolin (Reimann, 1931) was also effective not only in healing wounds but in thickening the skin of wounds which had been poorly covered. In 1932 Birnbaum reported the successful use of 1 in 10,000 thiocresol in burns and indolent ulcers, and as a dressing following skin grafting. These compounds, though not so expensive as glutathione, are still somewhat difficult to obtain. In 1933, however, Brunsting and Simonsen reported favourable results in 200 cases of cutaneous ulcer treated by means of a 0.5% solution of the sulphydryl compound cysteine. Cysteine, in the form of its hydrochloride, is stable, though cysteine alone soon breaks down into the disulphide cystine. A fresh solution of cysteine was therefore made up each day (6.35 c.cm. of normal NaOH to 1 gramme of cysteine hydrochloride in equal parts of saline and distilled water) and the dressing changed frequently. In the experimental field Owen (1937) found cysteine to be effective in accelerating the healing of radon necroses in guinea-pigs, such ulcers being particularly intractable to other forms of treatment. Other –SH compounds, such as methionine, (Toennies, 1937), have also been reviewed, and confirm the previous findings as regards their effect on growth by mitosis. Among the proprietary preparations merthiolate (Eli Lilly and Co.) contains a "thiol" group as a mercuri-thiosalicylate.

My personal experience regarding the sulphydryl series is something as follows. It is well known that, clinically, the ulcers of burns are "weak" ulcers in which healing is particularly sluggish. Feeling that this phenomenon might be due in part to coagulation of the proteins by heat in place of their normal slow breakdown (with unmasking of –SH groups) such as might follow trauma, it was decided to test the effect on such wounds of some of the compounds previously mentioned. Preliminary experiments in rabbits showed that cysteine hydrochloride (as a 0.5% ointment in lanolin) gave a 30 to 40% acceleration of healing, by measurement of surface area, in large excised

wounds and a somewhat slower rate of healing in wounds caused by heat. In the case of burns the additional factor of malnutrition due to the resultant ischaemia may also be important. The 0.5% cysteine hydrochloride ointment was then tried out clinically on the skin "reactions" following deep *x*-ray or radium therapy, where often a good "control" wound was also present. In such indolent areas cysteine hydrochloride undoubtedly hastened healing and, used in a wound which is already granulating, is not painful. If the patient complains of pain, the cysteine may be made up fresh as a 0.5% solution in "buffered" alkaline phosphate or according to the method of Brunsting and Simonsen. Decubitus ulcers, burns, scalds, and traumatic epithelial defects also responded favourably to the ointment.

Conclusions

One feels therefore that the sulphydryl compounds offer a new and definite approach, backed by experimental evidence, in the treatment of the large wound where healing is retarded. If, however, the additional factor present in embryo extract (which stimulates migration in addition to mitosis) could also be isolated an even more effective weapon might be in our hands. The possible dangers accompanying inhibition of fibroblastic activity, thus allowing of greater epithelial growth, yet have to be proved, and may or may not be real. It is more than likely that substances of the aldehyde class—e.g., propionaldehyde (Medawar, personal communication)—may eventually be found useful in such situations as the cornea and in plastic work, where even slight scarring is a matter of importance. In other situations the application above the dressing of wool and a firm bandage can do nothing but good, since Twyman (1922) has shown that epithelium will proliferate vigorously under pressures at which excessive fibroblastic growth is retarded. Used intelligently and in conjunction, therefore, all these factors regarding the physiology of tissue growth may be put to our advantage. It is for such reasons that this paper has been prepared.

Summary

The broad principles of wound healing have been outlined, emphasizing in particular that, although migration of cells alone is sufficient for the healing of the small wound, larger defects require the additional factor of growth by mitosis.

Epithelial growth is probably opposed by fibroblastic proliferation, but until our knowledge is increased deliberate cellular inhibition for long periods of time may be dangerous

as regards tumour formation. Physiological stimulation, on the other hand, would appear to be free from such dangers.

There is much evidence to show that compounds of the sulphydryl (–SH) group tend to stimulate growth by mitosis.

Some clinical applications of sulphydryl therapy have been mentioned, and a plea is made for their wider trial at the present time.

References

Arey, L. B. (1932). *Anat. Rec.*, **51**, 299.
Baker, L. E. (1929). *J. exp. Med.*, **49**, 163.
Bentley, F. H. (1936). *J. Anat.*, **70**, 498.
Birnbaum, I. R. (1932). *Ann. Surg.*, **96**, 467.
Brunsting, L. A., and Simonsen, D. G. (1933). *J. Amer. med. Ass.*, **2**, 1937.
Carnot, P., and Terris, E. (1926). *C. r. Soc. Biol.*, Paris, **95**, 655.
Carrel, A. (1924). *British Medical Journal*, **2**, 140.
—— and Baker, L. E. (1926). *J. exp. Med.*, **44**, 503.
Drew, A. H. (1923). *Lancet*, **1**, 833.
Gray, A. A. (1937). *Basis of Tissue Evolution and Pathogenesis*, p. 36, Jackson, Glasgow.
Haddow, A. (1938). *J. Path. Bact.*, **47**, 553.
Hammett, F. S. (1929a). *Proc. Amer. phil. Soc.*, **68**, 151.
—— (1929b). *Protoplasma*, **7**, 297.
—— and Reimann, S. P. (1929). *J. exp. Med.*, **50**, 445.
Medawar, P. B. (1938). *Quart. J. exp. Physiol.*, **27**, 147.
Nakumara, T. (1930). *C. r. Soc. Biol.*, Paris, **104**, 191.
Owen, S. E. (1937). *Growth*, **1**, 130.
Rapkine, L. (1930). *C. r. Acad. Sci.*, Paris, **191**, 871.
Reimann, S. P. (1930). *J. Amer. med. Ass.*, **94**, 1369.
—— (1931). *Ann. Surg.*, **93**, 624.
—— and Hammett, F. S. (1929). *Proc. Soc. exp. Biol.*, N.Y., **27**, 20.
—— and Hall, E. M. (1936). *Arch. Path.*, **22**, 55.
Toennies, G. (1937). *Growth*, **1**, 337.
Twyman, E. D. (1922). *J. Missouri med. Ass.*, **19**, 257.
Verne, J., and Verne-Soubiran, A. (1938). *C. r. Soc. Biol.*, Paris, **127**, 1090.

Paper II. "Stimulation and inhibition of connective tissue in mice following injection of benzpyrene." (1941).

The brilliant series of experiments by Kennaway, Cook and co-workers in London which culminated in the isolation and synthesis of the active carcinogenic constituents of tar, not unnaturally raised hopes that the purified chemical agent would lead rapidly to the specific biochemical change in the living cell which gives to it the power of autonomous, malignant growth. Up to the present, and despite much interesting and suggestive work, this hope has not been fulfilled. In general, the hydrocarbons are rather inert substances, and any form of substitution in the molecule leads to diminution of carcinogenic activity. The only compounds which are readily formed - quinones, photo-oxides and peroxides - are almost devoid of that striking power which is possessed by the parent substances of regularly inducing both epithelial and subcutaneous new growths.

Early work by Haddow and Scott, together with experiments 'in vitro' by Voegtlin and others, demonstrated that the primary action of the hydrocarbons is of a growth-inhibitory rather than a growth-stimulating character. Indeed, by increasing the dosage, even tumours which have been induced by the hydrocarbons can themselves be made to regress. This, in conjunction with the fact that general retardation of body growth, as well as of tumour growth, in animals is concurrently obtained by injections of the hydrocarbons in high dosage, suggests a general depletion in the body of some substance which is essential for both normal and neoplastic growth. The recent work on sulphur metabolism to which reference has already been made may well illuminate this point.

In 1940, it was felt that the fundamental nature of the observation that the hydrocarbons are growth-inhibitory in their actions on the tissues, was deserving of further investigation; the question at issue being whether, in regard to tumour formation, the inhibition was of a specific or non-specific type. Would, for example, further inhibition of growth by a non-carcinogenic agent summate with the inhibition imposed by the specific substance and increase its carcinogenic power?

Chick embryo extract (already becoming difficult to obtain) was chosen as a physiological growth stimulant. From contemporary experimental/

/experimental work in tissue culture by Medawar at Oxford, propionaldehyde was selected as a non-toxic and reversible inhibitor of mesenchyme. Both the embryo extract and the propionaldehyde were repeatedly injected into two groups of mice which had received single subcutaneous injections of benzpyrene. The result was clear. Fewer tumours emerged in the propionaldehyde group than in the controls. The addition of embryo extract, on the other hand, slightly raised the tumour yield. Inhibition of growth, 'per se', therefore, is not of fundamental importance in the genesis of tumours by a hydrocarbon.

PAPER II.

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STIMULATION AND INHIBITION OF CONNECTIVE TISSUE IN MICE FOLLOWING INJECTION OF BENZPYRENE.

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SINCE the carcinogenic hydrocarbons themselves produce " an immediate, constant and long-continued reduction in the rate of growth " both in the intact animal (Haddow, Scott and Scott, 1937) and in the individual cell (Earle and Voegtlin, 1938), it is of interest to decide if the carcinogenic effect of such an agent is in any way affected by the addition of a second, though essentially reversible, inhibitor of growth. In the following experiment a stimulator and an inhibitor of growth were added respectively to critical doses of 3 : 4 benzpyrene in contact with the subcutaneous connective tissue of mice. Chick embryo was chosen as the growth stimulant, propionaldehyde being employed as a nontoxic and reversible inhibitor of mesenchyme (Pomerat and Willmer, 1939).

A further purpose of the same experiment was the testing of propionaldehyde for any tendency towards sarcoma production of its own, since it was desired to use propionaldehyde for other purposes in which such a property would be undesirable (Riley, 1940).

EXPERIMENT.

Two hundred immature male mice, four to six weeks old, of an inbred (Medical Research Council) strain were used in the experiment. These were divided into four equal groups—A, B, C and D.

Groups A, B and C each received in the right flank a single subcutaneous injection of 0·5 mg. benzpyrene dissolved in 0·1 c.c. sterile olive oil, the dose and solvent of benzpyrene being chosen from experiments by Peacock and Beck (1938), and calculated to be sufficient to produce tumours in only a proportion of the animals receiving injections.

After two weeks the animals in Group A received in the right flank further injections of 0·5 c.c. chick embryo extract twice weekly for 16 weeks. The extract was prepared by finely chopping six fresh 10-day chick embryos (the eyes having been discarded) in 36·0 c.c. Ringer-Locke solution and centrifuging for five minutes. The clear supernatant fluid was kept in the ice-chest until required and was used for injection purposes within an hour of extraction.

In addition to benzpyrene the animals in Group B received an injection of 0·5 c.c. $M/10$ propionaldehyde subcutaneously in the right flank twice weekly for 16 weeks. The $M/10$ solution of propionaldehyde was made up by dissolving 8·9 c.c. of the pure aldehyde in 1 litre of Ringer-Locke solution.

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A fresh solution was prepared each second week. Throughout the experiment the pure aldehyde and the solution were stored in full bottles in the ice-chest over a trace of calcium carbonate A.R. The injections of propionaldehyde were not begun until the thirteenth week after the injection of benzpyrene, in order to exclude any direct chemical action between the benzpyrene and the propionaldehyde. Thus the inhibiting action of the propionaldehyde was added to any previous effect on the part of the hydrocarbon about the time of expected tumour emergence.

Group C received a single injection of benzpyrene to act as a control to Groups A and B.

Group D received no benzpyrene; 0·5 c.c. $M/10$ propionaldehyde alone was injected subcutaneously into the right flank twice weekly for 16 weeks.

RESULT.

Mortality was highest in Group B (benzpyrene and propionaldehyde), and lowest in Group C (benzpyrene alone). At the twentieth week the average weight of the animals was as follows:

Group A (benzpyrene and embryo extract)	33·4 gm.
Group B (benzpyrene and aldehyde)	31·5 ,,
Group C (benzpyrene alone)	31·7 ,,
Group D (aldehyde alone)	30·7 ,,

As has been anticipated, tumours began to appear from the 12th to 16th weeks in animals which had received an injection of benzpyrene. As soon as the tumours had become easily palpable, the animals were killed and the growths examined histologically. The tumours from all groups were sarcomata of the same type, and no significant difference was observed between those from the stimulated and those from the inhibited groups. Only one tumour showed signs of necrosis; this was in Group C. Tumours arose in 42 per cent. of the animals in Group A, and in 36 per cent. in the control Group C. In view of the relatively small number of animals employed, this difference cannot be regarded as significant. A more marked feature of the result was the fall in the number of tumours in Group B in which propionaldehyde was injected about the time of expected tumour emergence—this fall being to 22 per cent. (i.e. 14 per cent. less than the number of tumours in the control group). In Group D (propionaldehyde alone) no tumours arose, the mortality

TABLE I.

	Mice without tumours.			Mice with tumours.	
	Died before 20th week.	Died after 20th week.	Survived 40 weeks.	Died.	Killed.
Group A: benzpyrene and embryo extract	14	5	10	5	16
Group B: benzpyrene and aldehyde	17	9	13	1	10
Group C: benzpyrene alone	16	3	13	1	17
Group D: aldehyde alone	14	11	25	0	0

eventually being 50 per cent. These results are set out graphically in Figs. 1 to 3, and in Table I. Figs. 1–3 also show the date of death of animals

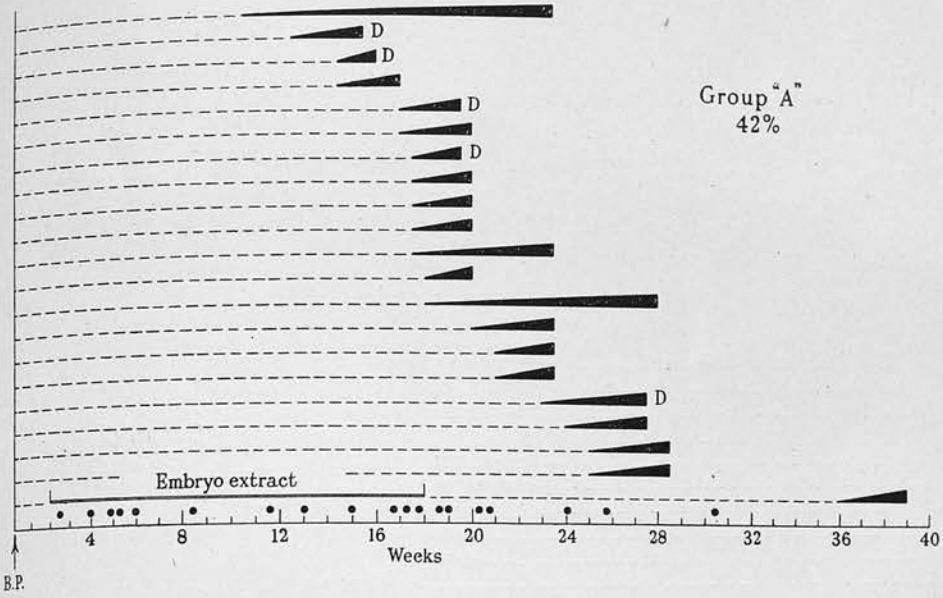

FIG. 1.—Tumours arising in 50 mice injected subcutaneously with 0·5 mg. benzpyrene; 0·5 c.c. embryo extract injected subcutaneously twice weekly from the 2nd to the 18th week. The dots mark the death of mice dying without tumour formation. The letter "D" denotes the death of an animal already bearing a tumour.

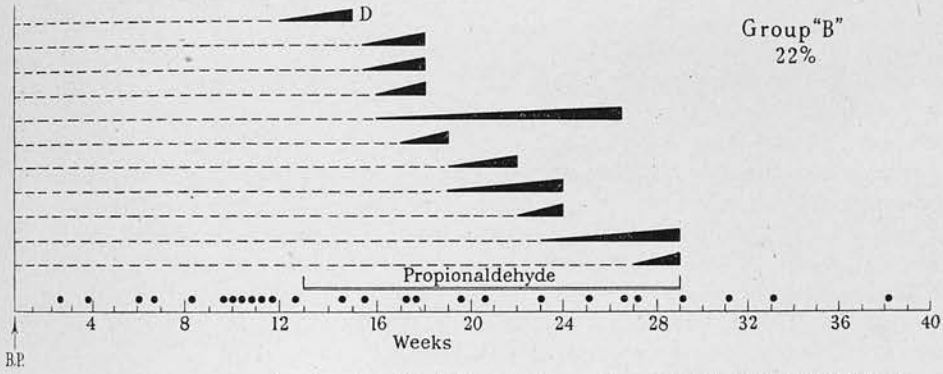

FIG. 2.—Tumours arising in 50 mice injected subcutaneously with 0·5 mg. benzpyrene; 0·5 c.c. *M*/10 propionaldehyde injected subcutaneously twice weekly from the 13th to the 29th week. Symbols as in Fig. 1.

which did not bear tumours. The remaining animals survived the period of 40 weeks over which the experiment was extended without bearing tumours.

DISCUSSION.

Following work by Heaton (1926, 1929) a factor was isolated by Medawar (1938) which in vitro gives rise to a specific, non-toxic and reversible inhibition of mesenchyme, without checking the growth of epithelium. Complete inhibition of all fibroblastic activity in vitro is believed by Pomerat and Willmer (1939) "to be a property of aldehydes as such. The reversibility of the effect has only been tested in the case of propylaldehyde." Glyceraldehyde would appear to be exceptional, since its effect on fibroblasts in vitro is not only to produce an inhibition of growth which is irreversible, but also, at similar concentrations, to check irreversibly direct sugar breakdown (glucolysis). No effect on glucolysis has been noted in the case of other aldehydes (Needham and Lehmann, 1937; Baker, 1938).

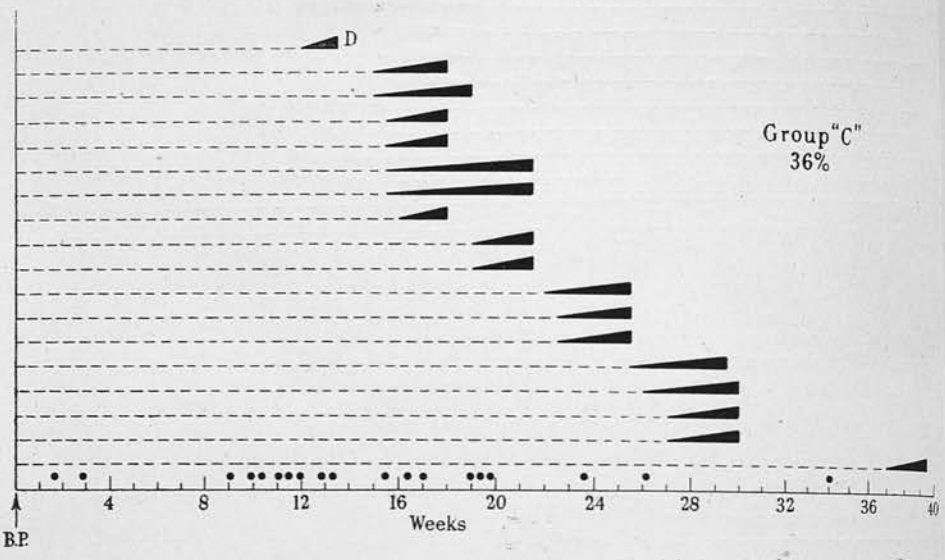

FIG. 3.—Tumours arising in 50 mice injected subcutaneously with 0·5 mg. benzpyrene only. Symbols as in Fig. 1.

It is thus possible that propionaldehyde is the active agent in Medawar's inhibitory factor, for similar specific and reversible inhibition of chick-heart fibroblasts in vitro has been noted by him in a plasma-saline medium containing concentrations of propionaldehyde ranging from $M/50$–$M/150$. Since in the living animal the aldehyde will soon be broken down by aldehyde dehydrogenases to its corresponding fatty acid, the injections in our experiment were made twice weekly with an $M/10$ solution.

However the results of the experiment are interpreted there was certainly no additive carcinogenic effect between the inhibition caused by the benzpyrene and the action of the propionaldehyde, the exact opposite being observed. In this way, therefore, propionaldehyde would appear to resemble heptaldehyde (Strong and Whitney, 1938), citral aldehyde, phloroglucinaldehyde and 3 : 4 dimethoxybenzaldehyde (Boyland and Mawson, 1938), which, in the

experimental animal, retard the growth rate, or even bring about complete regression of tumours which have already formed. It remains to be decided whether the effect of the propionaldehyde in reducing the yield of tumours was directed against the normal fibroblastic " stroma reaction," against some process connected with the malignant cells themselves, or against both. In this respect it may be significant that no further crop of tumours arose after the injections of the aldehyde had been stopped. In any case, it is evident that propionaldehyde itself can be absolved from any tendency towards sarcoma production in mice.

SUMMARY.

Under the conditions of the experiment, 0·5 c.c. $M/10$ propionaldehyde injected subcutaneously into mice twice weekly for the 16 weeks during which tumours from a previous injection of 0·5 mg. benzpyrene were to be expected, reduced the tumour yield from 36 to 22 per cent.

The injection of embryo extract twice weekly during the early weeks following a preliminary injection of benzpyrene had no marked effect on the tumour yield. The tendency was towards increase, rather than decrease, in the number of tumours produced.

Propionaldehyde alone appears to have no inherent properties for sarcoma production in mice.

This work was carried out in the Wilkie Surgical Research Laboratory, University of Edinburgh.

To Prof. Learmonth for his interest and to Mrs. Moore for her care in preparing the embryo extract, we wish to tender our best thanks.

REFERENCES.

Baker, Z.—(1938) *Biochem. J.*, **32**, 332.
Boyland, E., and Mawson, E. H.—(1938) *Ibid.*, **32**, 1982.
Earle, W. R., and Voegtlin, C.—(1938) *Amer. J. Cancer*, **34**, 373.
Haddow, A., Scott, C. M., and Scott, J. D.—(1937) *Proc. Roy. Soc.*, B, **122**, 477.
Heaton, T. B.—(1926) *J. Path. Bact.*, **29**, 293.—(1929) *Ibid.*, **32**, 565.
Medawar, P. B.—(1938) *Quart. J. exp. Physiol.*, **27**, 147.—(1940) *Proc. Roy. Soc.*, B, (in the press).
Needham, J., and Lehmann, H.—(1937) *Biochem. J.*, **31**, 1913.
Peacock, P. R., and Beck, S.—(1938) *Brit. J. exp. Path.*, **19**, 315.
Pomerat, C. M., and Willmer, E. N.—(1939) *J. exp. Biol.*, **16**, 232.
Riley, J. F.—(1940) *Brit. med. J.*, ii, 516.
Strong, L. C., and Whitney, L. F.—(1938) *Science*, **88**, 111.

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Paper III. "Retarding effect of glyceraldehyde on benzpyrene sarcoma formation in mice." (1944).

The reduction in the tumour yield following the injection of propionaldehyde, as already described, at once suggested further experimentation on this subject. Inhibitions of formed tumours had already been reported for several aldehydes by Boyland; while in tissue culture, Willmer and Wallensteiner had noted a general inhibitory effect of aldehydes on cell growth. Recently, Strong has reported continued studies with heptaldehyde (or heptaldehyde-bisulphite mixtures) on tumour inhibition, with varying degrees of success; though this procedure in other hands was found to cause renal damage as well as tumour regression.

To us, it seemed important to repeat our previous experiment using glyceraldehyde in place of propionaldehyde: first, since Mendel had recorded specific inhibition of tumour glycolysis by glyceraldehyde; and second, since glyceraldehyde is involved in the energy-yielding oxidation-reduction reaction of phosphorylating carbohydrate breakdown. It was in this very reaction that we had reason to suspect not only the participation of sulphydryl groups, but also the possibility that the reaction might be the site of the specific effect of the carcinogenic hydrocarbons.

The biochemistry of glyceraldehyde is somewhat complex since the compound exists in both monomeric and dimeric forms, and only the dextroisomeride of the racemic mixture is active in checking glycolysis. The result of the experiment, however, again showed inhibition of tumour emergence, the inhibition being essentially the same irrespective of the state of the aldehyde which was injected. The simplest explanation of the result appeared to be to accept Süllmann's hypothesis of aldol condensation of the non-phosphorylated aldehyde with the phosphorylated compound taking part in the glycolysis, thus forming condensed products which take no further part in biological processes.

While this explanation of the retarding effect of aldehydes on chemical carcinogenesis was tentatively accepted, the additional possibility was envisaged that aldehydes might themselves combine with sulphydryl compounds in the cell and thereby exert their anticarcinogenic effect. Such an action, if proved by further experiments, would be of great interest to the present line of thought that the fundamental process in chemical carcinogenesis in the cell is intimately correlated with the metabolism of organic sulphur.

PAPER III.

Retarding Effect of Glyceraldehyde on Benzpyrene Sarcoma Formation in Mice

J. F. Riley, M.B., F.R.C.S.Ed., and F. Pettigrew

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Retarding Effect of Glyceraldehyde on Benzpyrene Sarcoma Formation in Mice*

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(From the Wilkie Surgical Research Laboratory, University of Edinburgh, Edinburgh, Scotland)

(Received for publication March 23, 1944)

In an earlier experiment Riley and Wallace (8) showed that 0.5 cc. *M*/10 propionaldehyde injected subcutaneously into mice twice weekly for the 16 weeks during which tumors from a previous injection of 0.5 mgm. benzpyrene were to be expected, reduced the tumor yield from 36 to 22 per cent.

In tissue culture it has been demonstrated that the growth and cellular activity of chick periosteal fibroblasts and of the cells of a dibenzanthracene chicken sarcoma are both inhibited at similar concentrations by a large series of aldehydes (11). Before such a generalization for aldehyde inhibition *in vivo* can be accepted, it is necessary to test the effect on tumor emergence of glyceraldehyde, which differs from other aldehydes in three ways. First, glyceraldehyde is itself a normal intermediate of phosphorylating carbohydrate breakdown. Second, glyceraldehyde in crystals and fresh solution exists in dimeric (dimolecular) form and hence closely resembles glucose. Like glucose, dimeric glyceraldehyde has a similar action in inhibiting the initial step of phosphorylating glycolysis (1). Third, after being in solution for over 3 days glyceraldehyde is entirely converted into its monomeric (monomolecular) form, and as such is believed to be a specific inhibitor of direct sugar breakdown without phosphorylation. This last effect, the inhibition of glycolysis, is brought about by only one optical component, *l*-glyceraldehyde, of the racemic mixture (2, 5).

The inhibition by glyceraldehyde of anaerobic and aerobic glycolysis in tumor tissue was first pointed out by Mendel (4), and subsequently reported for chick embryo by Needham and his associates (6, 7). The question whether a special form of carbohydrate breakdown other than phosphorylating glycolysis is actually involved in the inhibition of glycolysis by glyceraldehyde has been raised by Macfarlane (3), although she was unable to offer a complete explanation of the specific inhibition of the *l*-isomeride. Süllmann (9, 10) has suggested that the inhibitory action of glyceraldehyde on carbohydrate breakdown may be due chiefly to fixation of triose phosphate by aldol condensation whereby compounds are formed that take no further part in the glycolysis.

In view of these remarks it was felt desirable to repeat our previous experiment, using glyceraldehyde in place of propionaldehyde.

EXPERIMENT

Two hundred immature male mice, 4 to 6 weeks old, of an inbred (Medical Research Council) strain were used in the experiment. These were divided into four equal groups: A, B, C, and D.

All four groups received in the right flank a single subcutaneous injection of 0.7 mgm. benzpyrene dissolved in 0.1 cc. sterile olive oil. Since the subsequent injection of glyceraldehyde was likely to bring about a reduction in the total number of tumors, a larger dose of benzpyrene was chosen than in the previous experiment in order to raise the tumor yield.

From the 11th to 27th weeks the animals in Groups A, B, and C received further subcutaneous injections twice weekly. Group A received 0.5 cc. *M*/10 "fresh" glyceraldehyde in Ringer-Locke solution; Group B received 0.5 cc. "old" glyceraldehyde in Ringer-Locke solution; and Group C received 0.5 cc. 0.9 per cent *d*-glucose in Ringer-Locke solution. Group D remained as a control. The *M*/10 solutions of glyceraldehyde were prepared by dissolving 0.9 gm. solid glyceraldehyde (Schering-Kahlbaum product) in 100 cc. of solvent and the fresh glyceraldehyde and *d*-glucose solutions were injected within an hour of preparation. The old glyceraldehyde solution was allowed to stand from 3 to 7 days before being injected. The glucose control was added in order to assess a possible pseudoglucose inhibitory effect of the fresh (dimeric) glyceraldehyde, and *d*-glucose was chosen since it is only in this form that glucose is utilized in tissue metabolism. It is from *d*-glucose that the normally occurring *l*-lactic acid (sarcolactic acid) is derived. All the animals were examined twice weekly for the appearance of tumors and the experiment was carried on for 40 weeks.

* Because of the difficulties of international communication the authors have not read proof of this article.

RESULT

As had been anticipated, tumors began to appear from the 11th week onwards in mice that had received an initial injection of benzpyrene. Autopsy was performed on any animal that died; mice bearing large tumors were killed and the tumors were examined histologically. Neither by loss in weight nor in general appearance did the mice in Groups A, B, or C show signs of toxicity as compared with those of the control Group D. The number of deaths in non-tumor-bearing mice in all groups was small and not more than 8 per cent of the animals in any group died before the period of aldehyde or glucose injections was completed.

The control Group D (benzpyrene only) yielded a total of 86 per cent of tumors, and of these 58 per cent had appeared by the 19th week. Group C (benzpyrene and *d*-glucose) yielded 78 per cent of tumors, of which 60 per cent had appeared by the 19th week. In Groups A and B (benzpyrene and aldehyde) the appearance of tumors was retarded, and although Group A (benzpyrene and fresh glyceraldehyde) gave a final yield of 72 per cent of tumors only 38 per cent had appeared by the 19th week. Group B (benzpyrene and old glyceraldehyde) showed the lowest tumor yield. This was 68 per cent, and of these only 36 per cent had appeared by the 19th week. No tumor in any group was seen to regress throughout the course of the experiment. The results are set out graphically and in the form of a table.

DISCUSSION

From the graph and table it will be seen that while the repeated injection of old glyceraldehyde produced the greatest degree of inhibition of benzpyrene sarcoma formation in mice, the curves of tumor emergence for both the fresh (dimeric) and old (monomeric) solutions of glyceraldehyde are so similar that no significant difference in the actions of the two solutions can be deduced. The repeated injection of *d*-glucose, on the other hand, gave rise to an initial acceleration in the appearance of tumors although the final yield was 8 per cent less than the number of tumors in the control group, which received benzpyrene only. Thus the action of both fresh and old solutions of glyceraldehyde resembles that of propionaldehyde in retarding the appearance of benzpyrene sar-

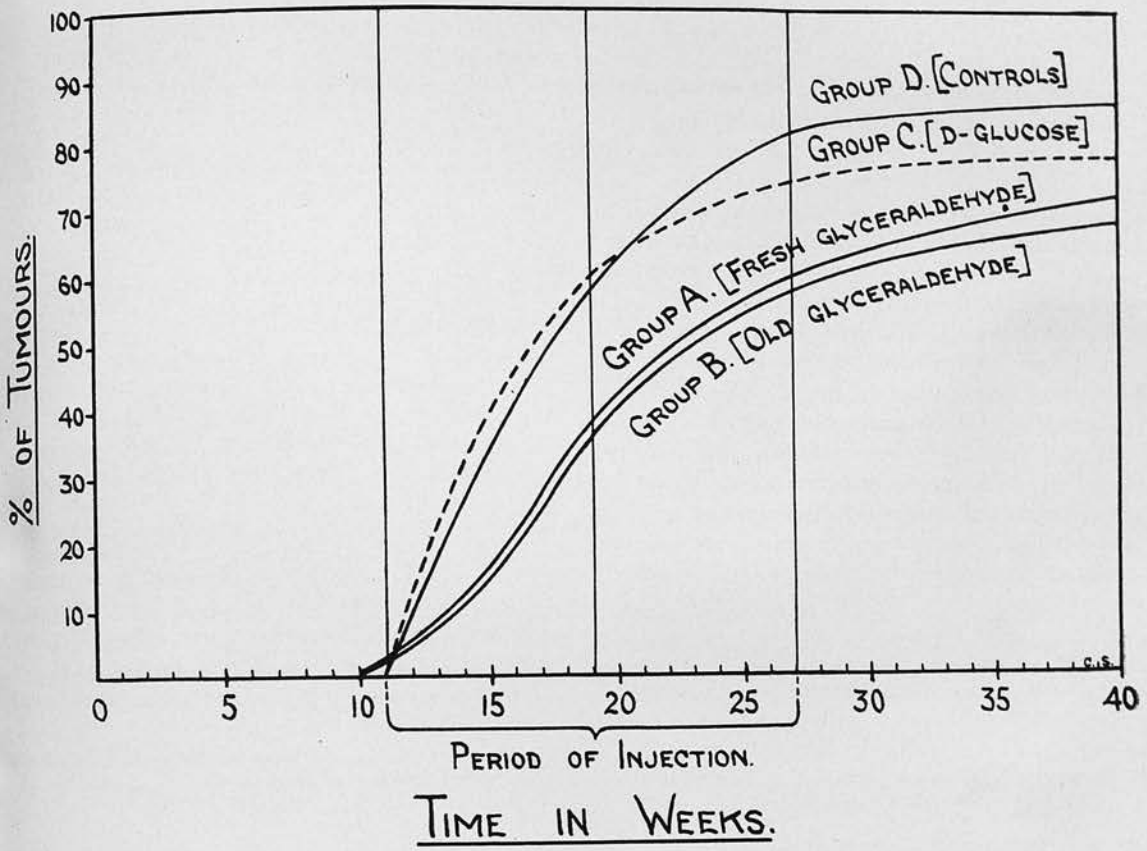

FIG. 1.—Delayed appearance of benzpyrene sarcoma, and slight reduction in tumor yield, after glyceraldehyde injections.

TABLE I

	Tumors before 11th week	Tumors from 11th to 19th weeks	Tumors from 19th to 27th weeks	Tumors from 27th to 40th weeks	Died without tumor	Survived: no tumor	Total percentage of tumors
GROUP A: Benzpyrene and fresh glyceraldehyde	1	18	11	6	5	9	72
GROUP B: Benzpyrene and old glyceraldehyde	1	17	11	5	7	9	68
GROUP C: Benzpyrene and *d*-glucose	0	30	7	2	2	9	78
GROUP D: Benzpyrene only	0	29	12	2	1	6	86
		period of injection					

comas in mice. It remains to be determined whether differences of this magnitude are significant.

SUMMARY

Under the conditions of the experiment, 0.5 cc. *M*/10 glyceraldehyde injected subcutaneously into mice twice weekly for the 16 weeks during which tumors from a previous injection of 0.7 mgm. benzpyrene were to be expected, resulted in a delay in the appearance of tumors and a slight reduction in the tumor yield. This effect was essentially similar whether "fresh" (dimeric) or "old" (monomeric) glyceraldehyde was used and resembles the inhibition, previously recorded in a comparable experiment, in which propionaldehyde was employed. It remains to be determined whether differences of this magnitude are significant.

We wish to record our gratitude to the British Empire Cancer Campaign for a gift of benzpyrene and to thank Professor J. R. Learmonth, of Edinburgh; Dr. H. Lehmann, of Cambridge; and Dr. B. Mendel, of Toronto, for their interest and advice.

REFERENCES

1. LEHMANN, H. Glycogen Breakdown in Muscle Extract and Yeast Juice. Nature, London, **141**:470-471. 1938.
2. LEHMANN, H., and NEEDHAM, J. The Action of Glyceraldehyde on Carbohydrate Breakdown. Enzymologia, **5**: 95-99. 1938.
3. MACFARLANE, MARJORIE G. The Phosphorylation of Carbohydrate in Living Cells. Biochem. J., **33**:565-578. 1939.
4. MENDEL, B. Krebszelle und Glycerinaldehyd. Klin. Wchnschr., **8**:169-170. 1929.
5. MENDEL, B., STRELITZ, FRIDA, and MUNDELL, DOROTHY. l-Glyceric Aldehyde and Tumor Metabolism. Science, **88**:149-150. 1938.
6. NEEDHAM, J., and LEHMANN, H. Intermediary Carbohydrate Metabolism in Embryonic Life. VIII. Glyceraldehyde and Glucolysis. Biochem. J., **31**:1913-1925. 1937.
7. NEEDHAM, J., and NOWINSKI, W. W. Intermediary Carbohydrate Metabolism in Embryonic Life. Biochem. J., **31**: 1165-1184. 1937.
8. RILEY, J. F., and WALLACE, A. B. Stimulation and Inhibition of Connective Tissue in Mice Following Injection of Benzpyrene. Brit. J. Exper. Path., **22**:24-28. 1941.
9. SÜLLMANN, H. Über die Hemmung der Glykolyse durch Glycerinaldehyd. Biochem. Ztschr., **296**:325-347. 1938.
10. SÜLLMANN, H. Die Hemmung der Milchsäurebildung im Muskelextrakt durch Glycerinaldehyd. Enzymologia, **5**: 372-382. 1939.
11. WILLMER, E. N., and WALLERSTEINER, K. The Effects of Certain Aldehydes upon the Growth of Cells "in vitro." Proc. Physiol. Soc., **96**:16-17. 1939.

Paper IV. "Acceleration by means of prolonged mechanical irritation, of carcinogenesis in the skin of mice painted with 1,2,5,6 - dibenzanthracene." (1945).

One of the oldest views concerning malignant disease is the theory - better, perhaps, the biological observation - that many human neoplasms occur at sites where chronic irritative processes have for long been at work. Yet of all forms of experimental cancer, no subject presents a more confused picture to the reviewers than that of the relationship of 'chronic irritation' to chemical carcinogenesis in the skin of mice. In 1939, from the many papers which had been published on this subject prior to that date, only the following tentative conclusions could be drawn by the writer:-

"(1) Preliminary irritation is probably without effect in hastening or retarding the appearance of tumours due to the later application of a known carcinogenic agent.

(2) Repeated thermal trauma inflicted during or after the application of a known carcinogenic agent probably hastens tumour emergence.

(3) No conclusions can be drawn from published experimental data as to the effects of repeated mechanical trauma inflicted during or after the application of a known carcinogenic compound."

Since 1939, two satisfactory reports have been published in which conclusions (1) and (2) have been verified under thoroughly adequate experimental conditions.

In 1943, it was decided to enquire further into the rôle of repeated mechanical (as opposed to thermal) trauma following the suboptimal application of a carcinogen. The result showed clearly that, under these conditions, tumour emergence is accelerated. The experiment thus tends to unify the conception of trauma as a co-carcinogenic factor; and emphasises that, in all probability, the train of events which leads to epithelial cancer following the application of a carcinogenic hydrocarbon is initiated very early in the induction period. Thereafter it would appear to be of small moment how further cell divisions are encouraged before a true tumour becomes manifest. This, together with the observation from earlier work, that tumours may emerge long after the single/

ngle application of a powerful carcinogen, gives indirect support to e view that it is the unchanged hydrocarbon which becomes fixed in the ll and then leads ultimately to the malignant transformation. From e work which has already been discussed, it is tempting to return to e suggestion of Fieser that a carcinogenic hydrocarbon may lodge in e protoplasm of the cell by opening up a protenoid disulphide linkage.

PAPER IV

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1945, Vol. XXVI, p. 63.

ACCELERATION BY MEANS OF PROLONGED MECHANICAL IRRITATION OF CARCINOGENESIS IN THE SKIN OF MICE PAINTED WITH 1 : 2 : 5 : 6-DIBENZANTHRACENE.

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From the Wilkie Surgical Research Laboratory, University of Edinburgh.

Received for publication January 24, 1945.

TWENTY years ago Deelman claimed to have observed the appearance of malignant tumours in wounds placed in the skin of mice which had been subjected to tar painting (Deelman, 1923–24). This observation led to many experiments on the relationship of trauma to carcinogenesis, though the results, when reviewed, appeared to be conflicting in the extreme (Eggers, 1932 ; Seelig and Cooper, 1933).

In many instances it is now apparent why decisive results were not obtained in these early experiments. Most of the investigations were carried out on small numbers of mice of mixed sexes and various strains. The tar was often of doubtful potency, and either the carcinogen or its solvent was apt to cause non-specific irritation, which can be avoided with appropriate doses of the pure hydrocarbons, provided they are not themselves too powerful to mask slight co-carcinogenic or anti-carcinogenic effects, and are applied at intervals so spaced that non-specific irritation to the skin is minimized. For this purpose acetone forms a more suitable solvent for the hydrocarbon than benzene (Orr, 1938).

Recently, two satisfactory experiments have been reported in which repeated trauma by means of heat markedly accelerated the appearance of tumours in the skin of mice which had received preliminary treatment with a carcinogenic hydrocarbon (des Ligneris, 1940 ; Lauridsen and Eggers, 1943). Des Ligneris, for example, states that if the thermal irritation is applied " at a time when only some of the animals have reached the cancer stage, it accelerates tumour production and cancerous change almost as effectively as the continuation of the specific treatment."

Such results stand in sharp contrast to the findings in some of the early experiments to which reference has already been made. Ludford, for example, who used small groups of animals, found that a weekly application of sandpaper to the skin of mice which had previously been tarred reduced the likelihood of subsequent tumour formation (Ludford, 1929). This worker agreed, however, that such a reduction in

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the tumour yield might have been due to the removal by the sandpaper of those cells which had already come under the influence of the tar. It was also noted that this form of irritation led to the development of subepithelial fibrosis at the site of injury.

The present experiment was therefore designed to inquire further into the effect of mechanical, as opposed to thermal, irritation of the skin of mice which had been treated with a suboptimal dose of a carcinogenic hydrocarbon.

EXPERIMENT.

Two hundred inbred male mice of the Medical Research Council strain, four to eight weeks old, were painted once weekly for 16 weeks with a 0·3 per cent. solution of 1 : 2 : 5 : 6-dibenzanthracene dissolved in acetone. Fifteen mice died during this period and no ulceration or papilloma formation occurred.

At the end of this time the mice were divided into two groups of 93 and 92 mice. The painted areas in the animals of Group "A" (93 mice) were then brushed twice weekly with a small brush of stiff bristle—a form of mechanical irritation which preliminary experiments had shown to be unassociated with surface ulceration. Group "B" (92 mice) remained as a control.

After 33 weeks of this treatment a clear result was obtained ; and as the numbers of mice which had died without tumour were not unduly high (17 mice in Group " A " ; 21 mice in Group " B "), it was decided to carry the experiment a stage further by stopping the mechanical irritation in Group " A " and by again painting both groups with the carcinogen. On this occasion the 0·3 per cent. solution of dibenzanthracene was applied twice weekly for a further sixteen weeks. The whole experiment extended over 75 weeks.

Sections of skin were taken from mice which died, and further sections were later obtained from those mice in both groups which survived the full period of the investigation. In making an assessment of the tumour yield only those papillomas which remained palpable for at least six successive weeks were taken into account, and for this reason one animal which developed a papilloma during the last weeks of the experiment was allowed to survive until this period had elapsed. Tumours were recorded as malignant when they became large, fixed or ulcerated, and each diagnosis was later reviewed in the light of histological examination. The results are set out in the form of a graph.

RESULTS AND CONCLUSIONS.

Papillomas began to appear in both groups of mice within two weeks of cessation of painting ; and for the next six weeks there was no significant difference between the two groups. Thereafter the numbers of both papillomas and carcinomas were significantly higher in the brushed group " A " than in the controls " B." At the 49th week 55 mice in Group " A " had borne tumours, of which 13 were malignant: while in Group " B " the number of papillomas was 36, of which only 4 had undergone malignant transformation. The additional factor of the mechanical trauma in Group " A " must be held responsible for this difference in tumour emergence in the two groups.

On the resumption of painting, fresh papillomas appeared in both groups of mice, while several pre-existing papillomas which had become stationary took on rapid and invasive growth. Despite the fact that there were now relatively more mice available for papilloma formation in Group " B," the tumour yield in this group 10 weeks after cessation of the second period of painting still lagged behind that of Group " A."

Indeed, at the 75th week the number of tumours in the controls had not quite reached the level of tumour production which the brushed group had previously attained at the 49th week, i.e. at the time when the second period of painting was begun. At the end of the experiment the total number of carcinomas (9) in the control group was also less than the number of carcinomas (13) in the brushed group at the 49th week. The brushed group finally yielded a total of 21 carcinomas. The failure to obtain more carcinomas in the controls during and after the second period of painting can

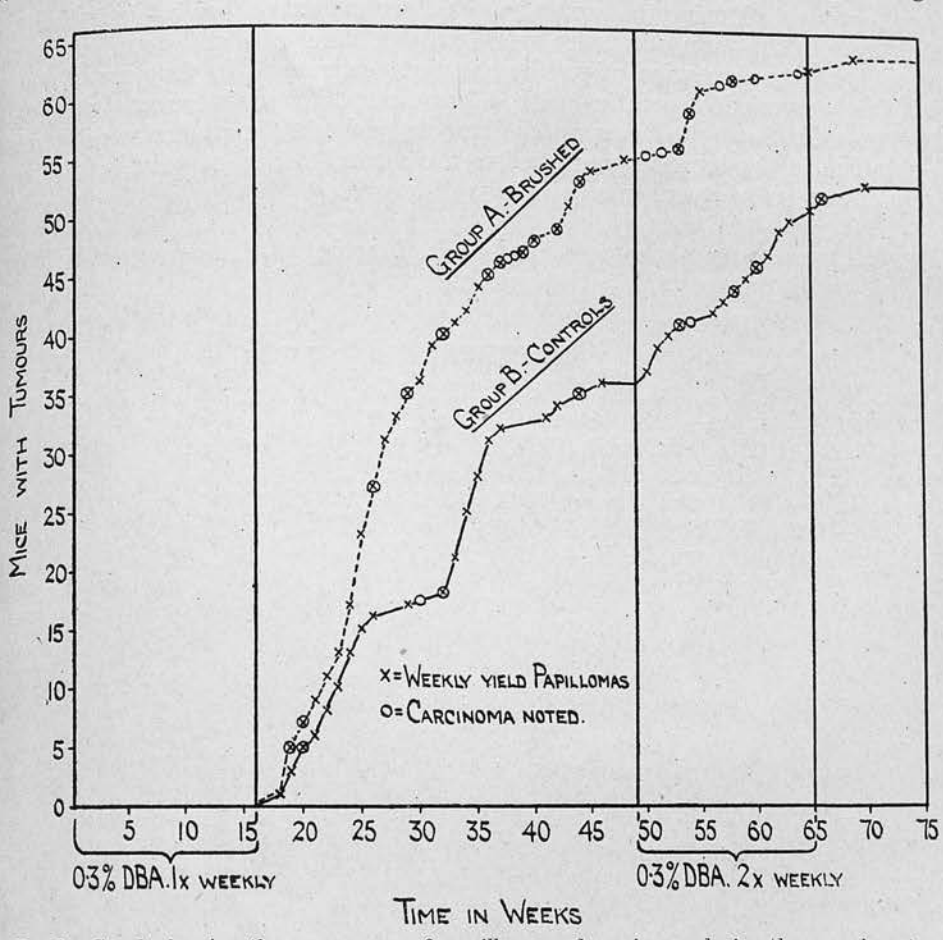

FIG. 1.—Graph showing the appearance of papilloma and carcinoma during the experiment.

be partly accounted for by the rapid death rate which took place from the 60th week onward in both groups of mice. Nevertheless there would appear to be no doubt that, under the conditions of the experiment, mechanical irritation by brushing following a suboptimal period of painting with dibenzanthracene markedly favoured subsequent tumour formation. This finding is thus in agreement with the results obtained by des Ligneris and by Lauridsen and Eggers in their experiments with thermal trauma inflicted under comparable conditions.

Finally, histological examination of sections of skin from the mice in Group "A" failed to show more marked subepithelial fibrosis than in the non-brushed group. It must be concluded, therefore, as Ludford had himself suggested, that the reduction in tumour formation which followed his application of sandpaper, in place of the present

method of brushing the skin, had led to destruction rather than to stimulation of the epithelial cells which had come under the preparatory influence of the carcinogen.

SUMMARY.

Two hundred young inbred male mice were painted with 0·3 per cent. 1 : 2 : 5 : 6-dibenzanthracene in acetone once weekly for 16 weeks, and were then divided into two equal groups. During the next 33 weeks, prolonged mechanical irritation to the painted areas in the form of a twice weekly application of a stiff brush significantly increased both the formation of papillomas and carcinomas in that group. At this stage a further period of painting with 0·3 per cent. dibenzanthracene twice weekly for 16 weeks did not quite bring up the tumour yield in the controls to the original level obtained in the brushed group before the second period of painting was begun. The significance of these results is briefly discussed.

We wish to record our thanks to Prof. J. R. Learmonth, University of Edinburgh, for his interest throughout this investigation.

REFERENCES.

Deelman, H. T.—(1923–24) *Z. Krebsforsch.*, **21**, 220.
Des Ligneris, M. J. A.—(1940) *Amer. J. Cancer*, **40**, 1.
Eggers, H. E.—(1932) *Arch. Path.*, **13**, 112.
Lauridsen, J., and Eggers, H. E.—(1943) *Cancer Res.*, **3**, 43.
Ludford, R. J.—(1929) *Brit. J. exp. Path.*, **10**, 193.
Orr, J. W.—(1938) *J. Path. Bact.*, **46**, 495.
Seelig, M. G., and Cooper, Z. K.—(1933) *Amer. J. Cancer*, **17**, 589.

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Paper V. Summary. "Experimental Cancer Research." (1944).

It is beyond the scope of a brief paper to attempt to summarise the whole of the achievements of experimental cancer research in the past; still less its hopes for the future.

Perhaps the best survey of experimental carcinogenesis in the past - the pathological basis for regarding malignant disease as an entity and the work on transplantable tumours - is to be found in Oberling's monograph, "Le problème du Cancer,"* published in Canada some two years ago. As regards "The Biochemistry of Malignant Tumours," the recent book of that name (Stern and Willheim, 1943) lists over 5,000 references and is itself so condensed that any further attempt at generalised compression would be sterile. A survey, in the most general terms, of those features of experimental cancer research which appear to the writer to be of lasting significance was recently compiled in the form of a lecture, a re-print of which forms the last in this series of papers.

The theme which has been running through this present thesis is that the probable point of attack of a carcinogenic hydrocarbon in the living cell lies in the intracellular proteins. The facts that certain tumours in animals can be transmitted by a cell-free agent, that such an agent appears to be comparable to the protein viruses, that recent work on the 'milk factor' in mammary cancer in mice again suggests transmission by a reproducible protein agent, and that the prosthetic groups of intracellular enzymes are attached to specific protein molecules, - all these suggest a bridgehead for further experimentation on cancer in the laboratory.

Yet in surveying the literature on this key subject of protein chemistry, certain deficiencies in our knowledge are at once apparent. The globular enzyme proteins preclude investigation by those methods, such as X-ray analysis, which have been so brilliantly successful in the case of the fibre proteins of slight biological significance. Determinations of 'molecular weight' and amino-acid content tell us little of the function of the parent protein in biological reactions; though, as in simpler systems, the new technique of employing tracer-doses of radio/

(* An English translation, by Woglom, is in the press)

/radio-active isotopes, may prove to be of value in this difficult field. From another angle, Scandinavian workers have attempted to gain insight into the nature and content of individual cell components by the use of ultra-violet absorption spectra; but here again, the quenching effect of the cell proteins may provide serious obstacles in obtaining the precise information which we desire. Somewhat unfortunately, the bulk of work in the past has been directed to a nuclear component which is more amenable to current methods of investigation - nucleic acid. A careful study of the literature leaves the reader in no doubt that reasoning has become strained in attempting to burden nucleic acid with properties and functions which a substance of such comparative simplicity can hardly bear.

It is here that new techniques are needed and new approaches would be welcome. The recent work of the Stedmans in the Edinburgh School on the proteins of the cell nucleus, though based on classical methods, may well offer scope for a renewed attack on the rôle of the proteins in both normal and neoplastic growth. "Cancer still awaits its Lister, its Darwin, its Einstein; and to one who can approach the cancer problem with new techniques and original ideas, it is a subject which still has great prizes to offer. Justification of past methods leads to hope in the future that cancer may join that growing list of human diseases which patient investigation has at length overcome."

PAPER V.

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EXPERIMENTAL CANCER RESEARCH *

By JAMES F. RILEY, F.R.C.S.E.

I AM very conscious of the honour in being asked to discuss with you for an hour or so this afternoon some of the problems and some of the results of experimental cancer research. Cancer, as you know, remains one of the outstanding—perhaps the outstanding—of the medical problems of the present day; for despite recent and very real advances in our knowledge concerning the ætiology, diagnosis and treatment of cancer, malignant disease still stands second only to cardiovascular disorders in the list of the great " killing diseases " of adult life.

As I see it, the cancer problem to-day—the problem of the ætiology of cancer—is in many ways comparable to the problem of hospital sepsis about the time of Lister, or of general biology about the time of Darwin; or, to take a more recent example, of astronomy before our own contemporary, Einstein: that is to say, that while we have acquired a very concrete body of evidence concerning cancer, we must admit that we still lack that unifying principle which will weld all these varying, and sometimes apparently divergent, facts into a single and coherent structure. Yet therein lies much of the fascination of experimental cancer research.

Sources of Knowledge concerning Cancer

Our knowledge of cancer comes from two sources. First and foremost stands observation—observation of the disease as it exists in man and animals. Secondly, since we have been unable to obtain all the information which we desire from observation alone, we have had to make appeal to experimental cancer research. Now we must always remember that good observation leads to good experiment; and we must never forget that ultimately the results of experiment must be reflected back to the disease as it afflicts mankind. That in itself provides sufficient justification for the clinical research worker; for we, as physicians and surgeons, are peculiarly favourably placed for appreciating and applying the knowledge gained by the academic research worker. Indeed, it is interesting to recall how many of the great advances in medicine have been made by practising physicians and surgeons.

Shall we, therefore, look first at the knowledge of cancer which has been gained by observation alone, and later follow up these observations in the experimental field to see whether experimental cancer research can amplify and illuminate the results of observation? Finally,

* A Guest Lecture delivered in the Class of Surgical Pathology, University of Edinburgh, 16th November 1943.

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and very briefly, let us attempt to sum up the interim position of the cancer problem as it exists at the present time.

Observation

(*a*) **Tar.**—One of the earliest and most important observations concerning cancer was made as far back as the year 1775 by that very astute surgeon, Percival Pott, for it was Pott who observed that cancer of the skin—and especially cancer of the scrotum—was particularly common amongst chimney sweeps. This observation lay dormant for many years, but toward the end of the last century other and similar observations came to link up with this curious fact. Workers in tar, pitch, shale-oil and certain industrial oils were likewise found to exhibit a high incidence of skin cancer. Many of the original observations on shale-oil cancer were made in the Lothians, and Dr Scott of Broxburn is a name which will always be remembered in this respect.

Diagram of Experimental Cancer Research

(For letters, see text)

Observation.	Experiment.
(*a*) Tar	+mice→carcinogenic hydrocarbons←
(*b*) X-rays	Similar precarcinogenic changes
(*c*) Genetic factors	Inbred strains of mice "high" / "low"
(*d*) Geographical factors	Carcinoma of liver→Azo-dyes
(*e*) Sex distribution	Carcinoma of breast→Oestradiol
(*f*) Tumour structure	Tumour grafts→Tissue culture (Compare normal tissues)
	(*g*) Filterable tumours (virus hypothesis)
	(*h*) The "milk factor" in mice

(*b*) **X-rays.**—Not only do chemical factors such as tar appear to play a part in the ætiology of skin cancer, but certain physical agents—X-rays, radium emanations and ultra-violet light—may also lead in time to a similar effect. Many of the pioneer workers in X-rays died from this cause; and the high incidence of lung cancer amongst certain cobalt miners in Bohemia has been shown to be due to the inhalation in the course of their work of small quantities of radio-active dust. It is important to remember, however, that in order to produce skin cancer, irradiation must be applied in small doses over long periods of time—a very different set of conditions from those under which irradiation is used in the cure of tumours, namely by large doses over short periods of time. This observation alone weighs appreciably against the idea of cancer being a "somatic gene mutation," for surely if this were true, during many therapeutic exposures *some* cell in the tissues should receive exactly the right quantum of energy, and hence ionisation, for mutation to take place. Yet I know of no instance in human beings in which this has been recorded.

(*c*) **Genetic Factors.**—The great advances during last century which followed the conception and proof of the bacteriological cause of so many diseases naturally led to a search for a parasite or an organism which might be the cause of cancer. Yet despite many and conflicting claims, none was found. Nevertheless, from such enquiries other and more important data have come to light, and in an examination of the statistical evidence concerning human cancer the suggestion arises that certain types of cancer may follow familial, hereditary or genetic lines. It is, of course, impracticable to carry out experiments in selective breeding in human stock, but occasionally this occurs under normal conditions. Thus, in some of the remote fjords of Norway, where inbreeding has taken place over many generations, we find significant differences in the cancer incidence in the various communities. Even more striking are the facts concerning tumours in uniovular twins. At least 26 cases have been recorded in which each twin developed a tumour of the same histological type at the same time and at the same site. It is of interest that there were no sarcomas in these paired tumours.

(*d*) **Geographical Factors.** — Not only do statistical figures suggest a familial incidence in some tumours, but there is a well-marked geographical distribution of certain types of cancer. Primary carcinoma of the liver, for example, is common in the Far East. In this country and in America this tumour is rare.

(*e*) **Sex Distribution.**—While cancer is equally common in males and females, there is a marked difference in the sites of predilection. In man the upper alimentary tract is most frequently affected. Women, on the contrary, tend to suffer from cancer of the generative organs. And here again there are differences. In unmarried and nulliparous women carcinoma of the breast is the more common tumour; in women who have borne children the cervix uteri is more frequently involved. We know, too, that the nulliparous woman—the woman who is anxious to bear children, but who fails to become pregnant—is frequently beset with menstrual disorders, and these in many instances we now believe to be due to endocrine disturbances.

(*f*) **Tumour Structure.**—Histological and pathological observations show that in general tumours arise from those tissues the cells of which normally divide—epithelium and fibrous tissue. Further, each tumour appears to consist of two parts. Just as a tree is composed of leaves set upon a trunk and branches, so the tumour consists of the tumour cells, the parenchyma, set upon a fibrous scaffolding which we call the stroma.

Here, then, is a list of data concerning cancer which have been obtained without recourse to a single experiment. They are interesting and at times suggestive; but they go no further. Now let us follow up each of these observations in terms of experimental cancer research, for if they are good observations they will lead to good experiments and we may hope thereby to amplify our knowledge of the cancer problem.

Experiment

(*a*) **Tar.**—It was only in the first decade of the present century that two workers showed that by painting the skin of mice repeatedly with crude coal tar malignant tumours could be produced. This seems rather an obvious experiment and we may well ask ourselves why this type of experiment had failed on so many previous occasions. Once the experimental conditions were known it was soon found that not all types of tar are active in causing tumours to appear, and not all animals are susceptible to the action of tar. Thus some workers had used the right sort of animals (mice), but tar which was later shown to be inactive. Others had used active tar but animals such as guinea-pigs, which are resistant to this agent. Still others had used both susceptible animals and active tar, but had failed to carry on their experiments for a sufficient length of time for tumours to emerge.

It will not have escaped your notice that since certain tars are inactive as regards tumour formation, other tars must contain "something" which is of high importance in tumour genesis. This point was investigated with great thoroughness by a British school of research workers. Kennaway, Cook and co-workers, by fractional distillation methods, finally isolated from gasworks tar the pure carcinogenic substances. On analysis these were found to consist of hydrogen and carbon in organic linkage and were consequently named the carcinogenic hydrocarbons. Later these chemicals were analysed, synthesised without the presence of tar, tested on mice and were found to be highly active inciters of both cutaneous and subcutaneous new growths.

How does a carcinogenic hydrocarbon act in determining the appearance of a tumour in the skin of a mouse? The first point to note is that a considerable period of time is necessary for a tumour to develop. On painting the skin of a mouse repeatedly for weeks with one of these agents, an early change appears to involve damage to the more specialised elements of the skin. Hair follicles and glands atrophy and some depilation occurs; the skin may become converted into a thickened sheet of stratified squamous epithelium. Next, the basal cells of the epidermis, or the cells of those follicles which have survived, become irregular in shape, size and staining properties. Small warts—papillomas—then appear on the surface of the skin until finally one of these warts grows rapidly, invades the deeper tissues and finally spreads by blood vessels and lymphatics to distant organs. A true tumour has developed.

(*b*) **X-rays.**—It is of interest that under the influence of small doses of irradiation over long periods of time the skin undergoes a series of changes comparable to those which are induced by a carcinogenic hydrocarbon. It was upon such data that Alexander Haddow erected his theory of tumour genesis. Haddow draws an analogy to the effect on colonies of bacteria of culture media which are made

progressively less suitable for growth. Under these conditions there finally emerges a strain of cells which are resistant to the inhibitory conditions of their surroundings. Haddow stresses the specific, prolonged and largely irreversible nature of the inhibition which is imposed on living cells by the carcinogenic hydrocarbons, and believes the ultimate appearance of a tumour to be a direct result of such inhibitory conditions. We hope that in time we shall be able to interpret this biological formulation of the cancer problem in terms of biochemistry, and recently a good deal of work has been done on such lines. Then indeed we shall be very close to the heart of the cancer problem.

(*c*) **Genetic Factors.**—Not only do different species of animals vary in their response to tar, but even susceptible animals show marked variations within the species. The possibility of hereditary susceptibility to certain tumours was largely investigated by Maud Slye. This worker was able to demonstrate that there are " high " and " low " strains of mice for various types of tumour and that such genetic factors apply both to tar-induced growths and to a neoplasm such as carcinoma of the breast which arises spontaneously in female mice just as it does in female human beings. Hereditary predisposition, of course, may be transmitted by male or female partner. At one time it was thought that the appearance of mammary cancer in human stock depended entirely upon such genetic principles. This view of mammary tumour formation, as we shall see, has had subsequently to be modified on at least two occasions by the demonstration of " extra-chromosomal factors."

Thus the early years of cancer research of this century were largely concerned on the one hand with the purification of the chemical material for experimental cancer production, and on the other with the refinement of the biological material for this type of work. With pure chemicals and pure inbred strains of mice, we are now in a position to carry out further experiments in which the number of variables can be reduced to a minimum and in which the value of the experimental results is correspondingly increased.

(*d*) **Geographical Factors.**—For many years the high incidence of primary cancer of the liver in the Far East was taken to be yet another example of hereditary differences ; but recent work has shed considerable light on this point. A year or two ago Japanese workers showed that by adding a chemical substance called " butter yellow " (an azo-dye stuff) to the diet of rats, many tumours of the liver could be produced. This was an important finding, for here was another tumour suitable for laboratory investigation and one which could be readily compared with its tissue of origin. When, however, the experiment was repeated in this country and in America, only an occasional tumour was observed. On looking into the matter more closely it was then found that the diet of cage animals reflects the food of the country. In Japan the rats lived on a diet of rice, supplemented usually with a slice of raw carrot ; whereas in the western hemisphere

cage animals receive a more balanced diet and one which contains an adequate vitamin content. When the experiment was once more carried out and " butter yellow " fed along with the Japanese diet, the original claims were substantiated. Moreover, by varying the diet in many ways it has been possible to demonstrate that a high degree of protection against the carcinogenic action of " butter yellow " can be conferred upon the rats, providing they receive in their food adequate amounts of casein (a protein of high biological value) and some of the vitamins of the B-complex. It seems reasonable to assume, at least on theoretical grounds, that similar protection could be conferred on the peoples of the Far East by suitable dietetic adjustments.

(*e*) **Sex Distribution.**—Carcinoma of the breast is one of the most important tumours, and one on which a great deal of experimental work has been performed. As we have seen, the work of Maud Slye laid stress on the genetic aspect of this particular tumour; but the differences which are observed in parous and nulliparous women led to further research.

In one experiment female mice of the " high " and " low " breast cancer strains were taken and the ovaries were removed in early life. Male mice were castrated to act as controls. It was then found that removal of the ovaries led to a decrease in the incidence of breast cancer in the female mice. Castration in the males was without effect, no tumours being observed as before. The corollary experiment was next carried out. Instead of removal of the ovaries, extra ovarian tissue was grafted into the female animals. The incidence of breast cancer then rose, and tumours were observed even in castrated male mice which had received implants of living ovarian tissue. Crushed ovarian tissue gave a similar result. Finally, the œstrogenic hormone, œstradiol, was isolated, analysed and tested for carcinogenic effect by repeated injection into mice. From the increase in the number of breast tumours which was observed, the relationship of the follicular hormone to mammary cancer in these animals became firmly established. The comparable doses used in these particular experiments are, however, far greater than those which are employed when œstradiol is administered for therapeutic reasons in human beings, and no great dangers are to be anticipated from carefully regulated hormonal therapy.

A most interesting piece of information now came to light. It was found that, chemically, the carcinogenic hydrocarbons and the œstrogens are related to one another and that their physiological properties overlap. Moreover, these substances are related to that important body of naturally occurring compounds, the sterols; substances which the experimental embryologists have shown to be of paramount importance in the processes of normal development of growth. For a time, at least, it looked as if here was the signpost to success in tumour research. Indeed, this may still be so, but the problem has become more complicated of late by the demonstration

both of highly active carcinogenic and œstrogenic substances which are not closely related to the normal body sterols. Nevertheless, this aspect of cancer research must continue to command attention, and the implications which are raised by this line of work are being diligently pursued.

(*f*) **Tumour Structure and the Behaviour of Malignant Cells.**—In the early days of cancer research and before the discovery of carcinogenic substances such as tar, œstradiol and "butter yellow," the only way in which a tumour could be obtained for experimental purposes was to wait for a new growth to arise spontaneously in a colony of laboratory animals. Very early it was found that the tumour could be transmitted by living grafts to other animals of the same species and that, with suitable precautions, serial transplantation by grafting could be carried on indefinitely. The interesting fact then emerged that only the tumour cells themselves are malignant and that on each occasion a fresh stroma has to be supplied by the new host. Unfortunately, any resistance which can be raised against the transplantation of tumour grafts appears to be a failure on the part of the host to supply the stroma which carries nutrition to the tumour cells.

The attempt was then made to grow the tumour grafts outside the body in tissue culture—experiments which were singularly successful. The cells grow in rather an unorganised way, but by serial transplantation in tissue culture malignant cells have been kept alive outside the body for periods up to twenty-five years. Are these cells still malignant? If we draw up some of the cells from the culture into a syringe and reinject them into a suitable animal, we find that the answer is "yes." A tumour forms in the experimental animal and is identical with the original growth from which the cells were first obtained many generations previously. In other words, malignant cells "breed true."

How do these findings compare with those of normal cells? Under somewhat strict conditions, grafts of normal tissue can be transplanted from one animal to another; but sooner or later these grafts die out. Yet in tissue culture normal cells will grow almost as readily as tumour cells; and under rich conditions of culture it may be impossible on morphological, metabolic or cultural grounds to decide which cells are normal and which are malignant. But carry out the biological test, reimplant the mixed culture back into a suitable host, and the old differences reappear. Normal cells die out; malignant cells continue to grow without reference to the tissue, organ or individual of which they are nominally members. The only notable exception to this rule is seen when there is a biological need for the normal cells. Then they grow until this want has been fulfilled and only later revert from their growing phase into a state of fixity, differentiation and function.

We see, therefore, that experimental cancer research has already justified itself, and that each and every observation has been clarified by the results of experiment.

(*g*) **Filterable Tumours.**—It is at this point that the experimental cancer research goes a step further: when something is discovered for which there is no previous suggestion in observation alone.

Shortly before the last war a research worker named Peyton Rous was engaged in grafting a tumour which had arisen spontaneously in a Plymouth Rock hen and, in the usual way, Rous was propagating the tumour from one bird to another by means of living grafts of tumour tissue. One day Rous cut out a graft, crushed it, ground it with sand and filtered the " breis " in a porcelain filter through which no cell could pass and obtained thereby a water-clear filtrate. On injecting a few drops of this clear fluid into another hen a tumour began to grow within a few days of injection and was found to have reproduced exactly the histology of the original growth. Naturally, when one reads of an experiment of this type, the word which springs to mind is a " virus." Can there be a " cancer virus " ?

This, indeed, was Rous' hypothesis; and on publication of his results a search was at once made among other laboratory tumours to see if they too could be transmitted by means of a filterable principle. In general, the search was disappointing. Only one or two tumours of this type were obtained; they all occurred in fowls and were all sarcomas. Since then several more such tumours have been added to the list. There is a kidney tumour in the frog which is filterable: another agent causes warts to appear in one breed of rabbit, and in another these warts may become malignant. It is surely of deep significance that under these latter circumstances the " virus " can now be no longer recovered from the tumour. Recently, too, there have been claims for a filterable mammary tumour in the mouse. Even so, these growths form but a small minority of the known laboratory tumours.

This brings us up against a paradox. We have already seen that there is no epidemiology of cancer; yet here is experimental evidence which suggests that certain tumours may be transmitted by a virus-like principle. Moreover, up to this point there has been nothing to support the view that cancer can be transmitted from one animal to another by infection or contagion. Only in experimental procedures are these results observed. This problem, however, has been partly bridged from two sides. The first approach concerns the possible nature of the " virus." The viruses of certain plant diseases have been isolated, purified and even crystallised, and in each case the virus is found to consist of protein. These protein crystals may be stored and later redissolved and injected into another plant when they once more multiply and reproduce their characteristic disease. This puts us in mind of certain other biologically active proteins which exhibit comparable properties—bacteriophage on the one hand and enzymes on the other; for enzymes too may multiply by autocatalysis once the original molecule has been formed by an independent process. We are prepared, therefore, to think of the " cancer virus " more in

terms of biochemistry than of bacteriology. Yet the paradox remains, and the question as to how such an agent may be transmitted is still unanswered. Indeed, the failure to find this clue was rapidly leading to the rejection of the virus hypothesis as a general explanation of the tumour problem.

(*h*) **The "Milk Factor" in Mice.**—Within the last two years of cancer research a further piece of evidence has come to hand which not only suggests a virus-like principle but also adds a possible mode of transmission. Again this work concerns a tumour which we have twice discussed to-day—carcinoma of the breast in mice.

Bittner took new-born female mice of the "low" breast cancer strain—mice in whom few of the females might on genetic grounds be expected to exhibit breast tumours in later life—and suckled them with foster-mothers of the "high" breast cancer strain. Later, these animals which had been fed by foster-nursing developed many more breast tumours than had been anticipated. The corollary experiment yielded a comparable result. If "high" breast cancer females are nursed by "low" breast cancer mothers, they in turn develop fewer tumours than a knowledge of genetics would lead one to expect. It seems reasonable to conclude, therefore, that "something" is passed in the mother's milk to the offspring where it lies dormant for several years and finally plays some determining rôle in the appearance or non-appearance of breast cancer. It is as yet too soon to assess fully the implications of Bittner's experiments, but again we tend to think of this work in terms of a "virus" and again turn to protein chemistry for a further elucidation of this point.

Conclusions

How, then, shall we attempt to sum up the position of the cancer problem as it exists to-day, and in which directions shall we look for further advances in our knowledge of this widespread disease?

We have seen in our brief survey that at least two tenable hypotheses present themselves as to the possible nature and cause of cancer. The first, Haddow's view, stresses the prolonged, possibly specific and largely irreversible inhibition of growth which many of the potent carcinogens impose on living cells before malignant transformation takes place. The biochemical changes which occur during this preparatory phase may well lead us to the cause, and ultimately the cure, of cancer. The second hypothesis postulates a "virus" as the proximate cause of cancer, and we have discovered good grounds on which the conception of a "cancer virus" may be approached by way of enzyme chemistry. This is a step which tends to unify the two hypotheses.

Yet perhaps the chief conclusion which we must draw from any survey of experimental cancer research is indeed a salutary one. All too often have we attempted to interpret the behaviour of the malignant

cell without that essential base-line, or yardstick of measurement, a sound knowledge of the processes which underlie *normal* growth, regeneration and repair. It is here that we look to the experimental embryologist, the worker in tissue culture, and to that form of tissue culture with which we all have to deal—the healing wound—for further data concerning both normal and neoplastic growth.

Recently, new techniques—the electron microscope, radioactive " tracer doses " and, above all, advances in protein and sterol chemistry —have all made signal contributions to our knowledge of biological systems ; for science advances on a broad front, and there are few subjects which have nothing to add to our more particular enquiry. Good observation, as we have seen, does lead to good experiment, and there are many observations and many experiments still to be made. Cancer still awaits its Lister, its Darwin, its Einstein ; and to one who can approach the cancer problem with new techniques and original ideas, it is a subject which still has great prizes to offer. Justification of past methods leads to hope in the future that cancer may join that growing list of human diseases which patient investigation has at length overcome.

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3rd April, 1945.