THE ISRAEL ACADEMY OF SCIENCES AND HUMANITIES

PROCEEDINGS · VOLUME I No. 5

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Jerusalem 1963

Published for the Israel Academy of Sciences and Humanities
by the Magnes Press, the Hebrew University, Jerusalem

Printed in Israel
At the Central Press, Jerusalem

Proceedings of the Israel Academy of Sciences and Humanities, Vol. I, No. 5

ON ATTEMPTS TO ELUCIDATE THE ORIGIN OF LEUKAEMIA FORMATION

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I. BERENBLUM

ACADEMICIANS of the 18th century were still able to take a lively interest in all phases of intellectual pursuits; we of the 20th century are no longer in this happy position. Any scientist who tries today to explain and interpret his work before a wide audience of scholars such as this, suffers from a feeling of frustration almost as acute as that engendered by linguistic barriers. (Some of us suffer from the double frustration.)

The present lecture is concerned with a specialized field that lies at the frontier of knowledge in a highly technical branch of medical research. One may say, of course, that all worthwhile research lies at the frontier of knowledge. But this does impose the necessity to preface any lecture on such a subject with a lengthy account of the whole field, of which the specialized subject forms but a small part.

I shall, therefore, have to begin with a general description of the cancer problem and how it is investigated experimentally, and follow it with a brief summary of recent researches on the causes of cancer and their mechanism of action. Only then shall I be in a position to approach the real subject of my lecture, which is "On attempts to elucidate the origin of leukaemia formation".

Cancer is a unique disease, or to be more exact, a unique group of diseases:

In its origin, it differs from inflammatory diseases in that it can arise spontaneously (that is to say, without any external influence); yet it can also be induced artificially — by certain chemical substances, by some forms of radiation, and in certain cases at least, by viruses, but interestingly enough, *not* by bacteria or other microorganisms.

In its early development, cancer differs from most diseases by its extraordinary long latent period which, in man, may be as long as 20 or 30 years, during which time no symptoms are usually detectable.

When *symptoms* finally appear, they are more elusive and diverse than those of almost any other type of disease.

In its general behaviour in the body, cancer differs from most active diseases in that the body shows little, if any, evidence of reacting against it. (Hence, it must be uprooted by surgical or other means.)

In its behaviour at the cellular level cancer differs, for instance, from degenerative diseases, in that it displays an *excess* instead of a *deficiency* in cellular activity.

And finally, *in terms of frequency of the disease*, cancer represents one of the commonest causes of death in man, taking only second place after cardiovascular diseases.

These features are, in themselves, sufficiently challenging to account for the fact that so much attention is nowadays paid to cancer research. But there is also another reason: Cancer represents an aberration of normal living tissue, and it is generally felt that if we could fathom the mystery of what makes the cancer cell behave the way it does, we might get closer to an understanding of the fundamental properties of the normal living cell.

As I have already mentioned, cancer is not a single disease. Its location in the body, or more specifically, the cell type from which it originates, determines its character and behaviour. A cancer of the brain is, for instance, a very different kind of disease from that of the skin; or a cancer of the lung, from that of the stomach. Furthermore, the term "cancer" denotes that it is a *malignant* tumour. There are also *benign* tumours, which are clinically far less dangerous and often quite harmless, but which are theoretically of great interest, serving as simplified models of the malignant forms.

A malignant tumour differs from a benign tumour in that it invades and destroys the surrounding, normal, tissues; and even more important, that it tends, after a time, to produce secondary centres of growth — or "metastases" — in distant parts of the body. The way this happens is that, by invading the surrounding tissues, some of the malignant cells break away from the main mass, and become transported, *via* the blood or lymph stream, to distant regions, where they continue to grow. The reason why the development of a metastasis is so serious a complication of a malignant tumour, is that the chances of a successful cure, by the conventional methods of surgery or radiation therapy, become greatly restricted, once such metastases have developed. (Hence the importance of *early* diagnosis.)

Cancer is, of course, no new disease: it has been known for thousands of years. Nor is it peculiar to man: almost every animal species may suffer from it. What is new is the method of studying the disease. For

centuries it was only known by its clinical manifestations. The modern approach is by animal experimentation.

The advantages of using animals for the study of the cancer problem are manifold:

(1) The process of cancerization is much more rapid in animals than in man, the latent period lasting, for instance, 4–18 months in mice, as against 5–30 years in man.

(2) When a cancer is artificially induced in an animal, the beginning of the induction process — the "zero hour" — is, of course, known precisely, so that the subsequent course of the disease, during the symptom-free "latent period", can be followed and carefully studied, despite the fact that the disease is clinically not recognizable at that stage. In man, on the other hand, the "zero hour" can only be approximately extrapolated in a statistical fashion.

(3) The scope and variety of experiments that can be performed in animals are almost unlimited, thus permitting all sorts of "questions" to be asked and "answers" to be obtained; whereas in man, experimentation is, of course, forbidden on ethical grounds (unless the experiment happens to have a reasonable chance of being itself beneficial, or at least harmless, to the patient).

(4) In the case of animals, there is a wide range of species in which the disease can be studied, so that *fundamental characteristics* of the disease, common to all forms of life, can be distinguished from *group characteristics*, restricted to particular species.

(5) Animals can be bred in such a way as to produce genetically inbred strains, all the individual members of the strain being as closely similar to one another as are identical twins in man. This permits much more exact work, with more reliable interpretations, than is possible in the case of mixed populations. It also enables one to recognize certain features, such as hereditary influences, *in an exaggerated form*, as I shall explain later.

When one considers all these advantages of animal experimentation, and also takes into account the great advances in scientific techniques of recent years, one realizes how ineffectual were the efforts of cancer research workers of, say, 50 years ago, as compared to now, and how much more promising the outlook is for the future.

As for the scope of cancer research, it is easy enough to define what are its *practical* objectives. They are: (1) to discover improved methods of *diagnosis*, so that the disease may be recognized, if possible, before metastases have developed, thus enabling the present methods of treatment to stand a better chance of succeeding; (2) to discover improved methods of *treatment*, so that the disease might be cured even when

the diagnosis comes late; and (3) to discover possible methods of *prevention*, so that the disease might not arise at all, or at least, might be kept in check during the latent period, thus avoiding altogether the necessity for treatment.

These practical objectives are, however, goals for the future. What is needed in the meantime is more knowledge about the fundamental nature of the disease and its mode of origin. We cannot seriously consider preventive measures, without knowing much more about the *causes* of the disease; nor can we expect to discover the ideal cure, without first acquiring more fundamental information about *the subtle differences between the cancer cell and the normal cell* — about which we still know so little.

This is the reason why so much of modern cancer research is still devoted to fundamental problems, carried out in scientific laboratories, remote from the hospital and the suffering patient. This also explains, perhaps, why applied research in the field of cancer has, so far, proved relatively unproductive, in contrast to the fundamental research of the past 30 years or so, which has made steady but very substantial progress.

I shall discuss only one facet of these fundamental studies, namely, that concerned with *the causes of the disease.*

The question of the "causes" of cancer raises two distinct problems: (1) how does the disease arise *spontaneously*? and (2) what are the extrinsic factors in the case of *induced* tumours, and how do these extrinsic factors operate? I intend to say only a little about the first, and go into more detail about the second.

With genetically inbred strains of mice, each strain has a different pattern of spontaneous tumours. In one strain, the spontaneous incidence of breast cancer may be very high; in another strain, that of lung cancer; in yet another, that of liver cancer, or of leukaemia, etc. Some strains are subject to several types of spontaneous tumours; others are almost entirely free from any type. All this shows that hereditary factors do play a decisive role in spontaneous tumour development, and that different genetic factors are involved for the different tumour types. The fact that heredity plays a role in the spontaneous development of tumours, is difficult to recognize in man or in mixed populations of mice, but becomes easily recognizable in inbred strains, as a consequence of genetic segregation.

It was soon found, however, that the genetic influence was, in most cases, only an expression of a "disposition", and that other factors played as important, if not more important, a role in determining the development of the tumour. These other factors comprise either a

persistent disturbance in the hormonal balance in the body, with some types of tumours; or the presence of specific viruses (some of them transmitted from mother to offspring), in others; or a combination of both. Indeed, there are probably many other *intrinsic* factors, which have not yet been identified.

Regarding the induction of tumours by *extrinsic* factors, the situation seemed at first more simple and straightforward. It had been known for a long time that cancer of the skin in man was liable to develop from long-continued contact with coal-tar, or as a consequence of prolonged exposure to ultraviolet light or x-rays. The same was shown to occur in animals, whether of inbred or mixed stock, and under such experimental conditions, the extrinsic agent seemed to be the over-riding factor.

During the past 30 years, much research has been carried out in the search for natural and synthetic "carcinogens" — as these extrinsic, cancer-inducing, agents are called. We now possess some 600 carcinogens of known chemical composition, as well as various physical means of inducing tumours in animals. But not all of them work the same way.

The action of some of them is at the site of application or injection. When such carcinogens are painted repeatedly to the skin, they produce skin tumours; when injected under the skin, they produce tumours of the subcutaneous tissues — called "sarcomas"; and when injected directly into organs, they produce specific tumours of those organs. Such locally-acting carcinogens belong mainly to the group of "polycyclic aromatic hydrocarbons" — of which 3:4-benzpyrene (a constituent of coal-tar) is a typical example.

Other carcinogens exist which do not act at the site of application or injection, but which induce tumours in specific organs of the body, irrespective of the route of administration. For instance, beta-naphthylamine produces tumours only in the urinary bladder; 4-dimethylaminoazobenzene, only tumours in the liver; urethane, mainly tumours in the lungs (though it possesses other interesting properties which I shall describe later); while 2-acetylaminofluorene produces a wide range of tumours, *all remote from the site of injection.*

It soon became apparent that the mere collection of new carcinogens, and of information about their *sites* of action, was not sufficient to advance very much our knowledge about carcinogenesis. What was more important was to understand their *mode* of action. The question was not only *what* produced tumours, and *where* these tumours arose, but also *how* precisely the causative agents acted.

We might consider first the relatively simple case of tumour induction

in the skin of mice, resulting from long-continued, weekly applications of a chemical carcinogen, such as 3:4-benzpyrene. (The case is called "simple" in the sense that no additional factors seem to be required.) The tumours, resulting from such action, begin to appear at the site of application after an average latent period of about 16 weeks. What happens during these 16 weeks?

When the treated skin, during this latent period, is examined microscopically, certain changes are observed in it which denote increased cellular activity. But identical changes are also observed in skin treated with any kind of mild irritation. These changes are, therefore, probably mere side-effects, unconnected with the carcinogenic process. Yet, something specific must be happening during the latent period, to account for the eventual development of the tumours. Since microscopy did not help in answering this question, non-morphological, indirect methods — by "experimental tricks" — had to be devised to provide a clue.

When the carcinogen was applied once only, tumours failed to appear. But when, after such a single application of a carcinogen, another agent, croton oil — which is itself a very weak carcinogen — was applied repeatedly to the same area of skin, tumours appeared in large numbers. Furthermore, when the experiment was performed in reverse, that is to say, with the croton oil treatment given *before* the single application of benzpyrene, tumours virtually failed to appear. It was not even necessary to use a carcinogen such as benzpyrene for the initial application; urethane (which is not carcinogenic for the skin), followed by croton oil, proved equally effective.

We were thus led to the conclusion that a sequence of independent processes was involved during the latent period, consisting of (1) a rapid "initiating" process, followed by (2) a slow "promoting" process; the two being required to operate in this particular sequence.

These findings, derived from our own experiments, were in keeping with certain results of others, based on a different set of experiments, that led to the formulation of the "two-stage mechanism" hypothesis, which postulated that the first step in the process of skin carcinogenesis (the "initiating" process) represented an irreversible change from normal cells into "dormant" tumour cells, and that a very different action (the "promoting" process) was responsible for the encouragement of these "dormant tumour cells" to develop into progressively growing tumours.

The two-stage mechanism hypothesis does not, of course, provide a complete explanation of what takes place during the long latent period of carcinogenesis. But it does indicate the existence of a kind of *bio-*

logical chain reaction, in contrast to the earlier conception of a slow, continuous, *single* process.

That things were, in fact, more complicated than that originally visualized by the two-stage mechanism hypothesis, became evident when one began to consider other systems of carcinogenesis.

It had long been known that with some types of tumours, a specific virus was involved, and that such a tumour could be reproduced in other animals by transfer of the particular virus. What, then, was the function of viruses in carcinogenesis in general, and what role did they play in chemical carcinogenesis in particular? It was also known that, with certain types of tumours, a disturbance in the hormonal balance in the body was critical for the development of that tumour. Did the hormones in question act as "initiators" or as "promoters", or was their action independent of the "stages" of carcinogenesis? (My colleague, Dr. Haran-Ghera, is at present investigating this very problem.) Even in the case of skin carcinogenesis, it was found that urethane did not have to be applied to the skin itself. Injection of urethane at a distance, followed by local applications of croton oil to the skin, led to tumour development in the treated skin. How did this fit into the picture of the two-stage mechanism hypothesis?

For these, and many other reasons, it became imperative to try and apply the experimental procedure for detecting a two-stage mechanism to tumour-inducing systems other than that of the skin. This proved, for technical reasons, rather difficult. But my colleague, Dr. Trainin, and I did eventually succeed in doing this for leukaemia induction; and the present lecture is actually concerned with this phase of our work.

Before describing the results obtained, I should try to explain what is meant by "leukaemia", and also provide some background information about certain peculiar features of experimental leukaemia induction in mice.

When a tumour arises in a particular organ or tissue, it usually grows locally as an expanding mass of tissue. We speak of it as a "solid" tumour. But when a tumour arises from cells which are normally scattered throughout the body, in the form of circulating blood cells, the tumour itself is also dispersed. The condition is known as "leukaemia".

There are actually two major types of leukaemia, according to the two predominant types of nucleated blood cells. "Myelogenous leukaemia" is derived from the myeloid cells that originate in the bone marrow; "lymphatic leukaemia" is derived from lymphoid cells that originate from centres of lymphatic tissue. In mice, *lymphatic leukaemia* is the more common type; and it generally arises, in this species, in the thymus gland (located in the upper part of the chest), where the disease

may, at first, be localized before it becomes disseminated as the more typical, blood-borne, disease.

In some strains of mice, this type of lymphatic leukaemia develops spontaneously in 60–80 per cent of animals; in other strains, it is rare or practically non-existent. One can, however, induce the disease in strains in which it does not normally exist, by means of x-rays, by certain carcinogens, or by large doses of oestrogenic hormones.

The artificial induction of leukaemia (called "leukaemogenesis") presents certain peculiarities that distinguish it from other kinds of tumour induction (called "carcinogenesis").

For instance, for successful leukaemogenesis by x-irradiation in non-leukaemic strains of mice, 3 conditions are needed: (1) the radiation must be applied to the whole animal; (2) it must be given in divided doses, at intervals of about a week between each dose; and (3) the thymus gland must be functioning in the body. These conditions require further clarification.

When, for instance, adequate doses of radiation are given, but one limb of the animal is each time shielded with lead, leukaemia does not develop. Again, if after the radiation treatment without shielding, normal bone marrow is injected into the animal, leukaemia is once more prevented. It would appear, therefore, that the radiation performs two separate tasks: (i) the actual induction of the leukaemia, and (ii) the depression of bone marrow, which normally prevents the leukaemogenic process from manifesting itself.

As for the role of the thymus gland, this too is more complicated than might appear at first sight. If the thymus gland is first removed from the animal, and radiation treatment given subsequently, leukaemia fails to develop. But if after this procedure, normal thymus tissue is injected into the animal, leukaemia develops after all. This means that the thymus is essential for the final result, but need not be present at the time of the radiation.

There is yet one further complication about mouse leukaemia, namely, the involvement of a specific virus. Such a virus can be detected in those strains of mice which develop leukaemia spontaneously, but not in strains in which the disease is rare or absent. When this virus is extracted from the former and injected into newborn mice of the latter strains of mice, the disease tends to develop many months later. The interesting observation was made that if mice, which normally do not seem to possess the virus, are irradiated, the virus becomes detectable at or about the time when the *induced* leukaemia develops. This extraordinary result lends itself to several possible interpretations: (i) the virus may have been present all the time, but in insufficient amounts

to be detectable; (ii) the virus may have been present in an "incomplete" form, requiring radiation to convert it into a "complete", active, virus; or (iii) the virus may indeed have been absent, and subsequently "created *de novo*" by the radiation treatment (since viruses are, after all, chemically related to the normal genic material of the living cell, from which they may conceivably be formed).

I have, at long last, reached the point where I can begin to discuss the real subject of this lecture, which is concerned with an attempt to clarify some of the many complications of leukaemogenesis in mice, by applying the two-stage mechanism principle.

This phase of the work arose out of an observation from the literature, a few years ago, that radiation leukaemogenesis in mice could be greatly enhanced by the simultaneous administration of urethane. Since urethane was known to play an important role in the study of *skin* carcinogenesis — serving there as a powerful "initiating" factor — it was thought worthwhile investigating whether its role in *leukaemogenesis* was also a manifestation of a two-stage process. If so, could it not be used as an analytical tool in the study of leukaemogenesis as a whole?

By repeating the double action — of radiation plus urethane — under modified conditions, we were able to show that the enhancing effect of urethane still operated when it was administered 2 weeks after completion of the radiation treatment, *but not when the sequence was reversed.* From these and other experiments, it became evident that a two-stage mechanism was indeed involved, *with urethane acting this time as the "promoting" factor.* The system even worked when the radiation was administered as a single dose, and then followed by urethane injections. This provided the "analytical tool" sought for.

The next phase of the work was an attempt to determine what each of the two actions — radiation and urethane — specifically contributed to the overall leukaemogenic process, and how the virus entered into the scheme.

An experiment was devised with the aim of inducing leukaemia by radiation plus urethane, but under conditions that the two actions could be investigated separately. Mice of a non-leukaemic strain were given a single dose of x-rays, and killed the following day. Various tissues from these animals were removed, minced and homogenized, and the various homogenates injected into separate groups of normal mice of the same strain. Urethane was subsequently injected into the recipients. The "experimental trick" in this experiment was that the irradiated animals did not receive any urethane, while the urethane treated mice were not irradiated; yet a link was created between the two by tissue extracts from the one being injected into the other.

The result of the experiment was that many of the recipients developed leukaemia; and it did not seem to matter very much which tissue had been used for transmission. Leukaemia did *not* develop in mice which received tissues from irradiated animals, *but were not subsequently treated with urethane*; nor did leukaemia develop to any significant extent in mice receiving tissues from *non-irradiated* mice and subsequently *treated with urethane.*

The conclusion reached was that a "transmissible factor" was rapidly produced in irradiated mice, but that this "factor" was itself incapable of inducing leukaemia when transferred to normal mice, unless the latter were subsequently given urethane.

In order to obtain further information about the nature of the "transmissible factor", the experiment just described was repeated with a slight modification — making the interval between the irradiation and the extraction of tissues a month instead of 24 hours. The result was virtually the same. This showed that the "transmissible factor" persisted in the body for a long time, and could not, therefore, be one of the more simple products of radiation damage in the body.

So far, the results were consistent with the notion that the "factor" might be a virus-like product. Much more evidence was needed, however, for actual proof of this. Even if it were "a virus-like product", it could not be identical with the actual leukaemia virus which, as already mentioned, appeared in repeatedly irradiated mice at the time of leukaemia development. In the first place, the present factor appeared within 24 hours of irradiation, instead of 6–8 months later; in the second place, it required the participation of urethane to induce leukaemia.

In a further experiment, the irradiation was performed on the isolated tissues *after these were removed from the body*, instead of *in vivo*, and the material then injected into normal mice, which were subsequently given urethane treatment. Once again leukaemia developed, though only with some of the tissues, not with all. Further experiments along these lines should enable us to determine more exactly in which organs the "transmissible factor" was liberated by the radiation. From the results so far available, it would seem that the brain, liver, thymus, and bone marrow, are equally effective in providing the "factor" on irradiation *in vitro*. It is not easy to visualize, at first sight, what these organs have in common.

The next logical step was to fractionate the irradiated tissues, by filtration, etc., in order to determinate the particulate size of the "transmissible factor", and thus to provide more convincing evidence as to whether it was indeed a "virus"-like entity. Such experiments are in progress, in cooperation with Dr. Hodes and Miss Boiato, but so far

no conclusive results are available. Another investigation, in cooperation with Dr. Cividalli and Dr. Hodes, is concerned with the nature of the factor in bone marrow, which inhibits leukaemogenesis.

Since then, results from other laboratories, where some of our work had been followed up, provided new information which introduced further complications. This deals with the leukaemogenic action of urethane itself.

While in *adult* mice, urethane alone is either not leukaemogenic at all or only mildly leukaemogenic, according to the strain of mice used, several investigators have recently found that in *newborn* mice, urethane alone is very potently leukaemogenic.

The possibility had, therefore, to be considered whether, in our original two-stage experiments, the radiation merely performed *in adults* what already existed naturally *in newborn*, namely, the reactivation of the quiescent, adult thymus gland into a more actively functioning form, similar to that of the infantile gland. According to this interpretation, urethane acts directly on the thymus, but the latter must be in a functioning state, and when it is not in a functioning state (i.e. in adults), prior treatment with radiation is needed to "activate" the gland.

However, in thus trying to provide a simplified explanation for the mode of action of urethane, one is merely complicating the other side of the picture. We already know that the role of *radiation* in the over-all system is complicated enough, involving a depression of the inhibiting effect of bone marrow on leukaemogenesis, and in the case of repeated radiations, the manifested liberation of an active virus which was not demonstrable before. It is now suggested that x-rays, in addition, "prepare" the thymus gland for the effective completion of the leukaemogenic process by urethane, while leaving the situation regarding the role of the virus more mysterious than ever.

Our alternative, tentative, explanation of the over-all process is that *in adult mice*, a single radiation liberates an "incomplete" or "inactive" virus, which is converted into a "complete", active, virus by the action of urethane (or, of course, by further radiations); whereas *in newborn mice*, the radiation is not needed at all, because in such young animals, urethane can presumably liberate the "incomplete" virus (as radiation can do in *adult* mice), and later convert it into a "complete" virus.

It should be possible, by suitably devised experiments, to discover which of the two alternative explanations is the correct one, and experiments are now in progress with this aim in view. Since each experiment of this sort takes about 18 months to complete, it will, therefore, probably take several years before we can expect to find the solution to this intriguing problem.

I should like, meanwhile, to describe one further experiment which deals with another aspect of the problem, already referred to.
I mentioned before that, when using repeated radiations only, it is essential that the thymus be present in the body, though not necessarily at the time of the radiations, for leukaemia to develop. According to our interpretation of the phenomenon, this is explained by the supposition that the cells of the thymus are the ones that become "infected" by the "complete" virus, but that other cells in the body are capable of performing the earlier function — of liberating the "incomplete" virus from which the "complete" virus is presumed to be derived.
This idea was tested by us, by irradiating mice from which the thymus had previously been removed; then transferring some of the tissues from these animals to normal mice, and subsequently giving urethane to the recipients (which had, of course, their thymuses intact). Leukaemia developed, as anticipated.

You might wonder what all these complicated experiments have to do with the development of leukaemia in man.
There is no doubt any longer that radiation can cause leukaemia in man, whether the source of the radiation is from excessive x-ray treatment or from greatly increased radioactivity in the environment. There is still some doubt whether a virus is involved in human leukaemia, as it is in mice, though a number of claims have been made in recent years, purporting to show that such a virus can be demonstrated in the human disease. The human form of lymphatic leukaemia does differ from that observed in mice at least in one respect, namely, that in the human form the disease does not originate in the thymus, as far as one can tell.
To what extent, then, are the experimental results, derived from mouse experiments, applicable to the elucidation of the mode of origin of human lymphatic leukaemia?
Experience of the past 30 or 40 years, from the study of experimental carcinogenesis, has convinced us that, as far as basic principles are concerned, the phenomenon is essentially the same in experimental animals as in man. There are, of course, differences in detail, but these are no greater than, say, the differences between mice and rats, or rats and rabbits. Indeed, from the viewpoint of carcinogenesis, the mouse is probably closer to man than to the rat and the rabbit, and certainly much closer than to the guinea-pig or the cat. One has, of course, to make allowances for these species differences, and to beware of the danger of drawing broad conclusions from limited sets of observations. This is what I had in mind when I tried to distinguish between "fundamental

characteristics" and "group characteristics" about cancer. The danger there was to generalize too freely from human evidence only; I am now stressing the opposite danger, of generalizing too freely from what happens in the mouse.

There can, however, be no doubt whatever that, when more is known about the disease in its various manifestations, experimental studies, such as those I described, will play their part in the final elucidation of the origin of leukaemia formation.

Read 18 December 1962