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METHOD FOR THE DETECTION OF CANCER

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ABSTRACT OF THE DISCLOSURE

A method is provided for determining whether cancer is present in a tissue. This method makes use of the bioluminescent reaction between ATP and firefly lantern extract and measuring the amount of light which is emitted. A comparison is made between cells taken from the subject which are known to be noncancerous and the cells which are thought to be cancerous. The presence of cancer is indicated by a significantly lesser amount of light being emitted by the sample which is suspected to contain cancer.

This is a continuation of S.N. 433,461 filed Feb. 17, 1965, and now abandoned.

This invention relates to a method for the detection of cancer. More particularly, this invention relates to the use of the bioluminescent reaction, between adenosine triphosphate (hereinafter referred to as ATP) and firefly lantern extract as a means for detecting the presence of cancerous cells.

It is known that the energy requirements for all biological reactions are directly or indirectly supplied through ATP which is present in all living tissue cells. It has now been found that the ATP content of cancerous cells is only ⅔ to 1/10, or less, the ATP content of normal cells.

It is an object of this invention to provide a method for the rapid detection of cancerous cells by monitoring the ATP content of the cells.

It is a further object of this invention to provide a means for the utilization of the bioluminescent reaction between ATP and firefly lantern extract as a means for detecting the presence of cancerous cells.

Firefly lantern extract contains a mixture comprising luciferin, luciferase and the cation, magnesium. When a material containing ATP is brought into contact with firefly lantern extract, there is a reaction accompanied by the emission of light. This emission of light is caused by the reaction of ATP with the constituents of the firefly lantern extract in the presence of oxygen. This phenomenon ies called firefly bioluminescence.

The reactants required for firefly bioluminescence are the substrate, luciferin; the enzyme, luciferase; the activator, ATP; a cation (usually magnesium); and oxygen. The overall reaction is an oxidation reaction catalyzed by the enzyme, luciferase, which results in the emission of light. A general mechanism of the course of the reaction is as follows:

(1)

$$\text{ATP} + \text{luciferin} \xrightarrow[\text{luciferase}]{Mg^{++}} \text{luciferyl adenylate} + \text{pyrophosphate}$$

(2)

$$\text{Luciferyladenylate} \xrightarrow[\text{luciferase}]{O_2} \text{oxyluciferyladenylate} + \text{light} + H_2O$$

This reaction is absolutely specific for ATP. The ATP may not be replaced by any other known compound.

The objects of this invention are attained, briefly, by a process which comprises the utilization of the bioluminescent reaction between ATP and firefly lantern extract. A sample of the tissue suspected to contain cancer is prepared, for example, by comminuting it and suspending it in distilled water. An equal amount of a sample of the adjacent tissue, such as obtained from the same animal and organ, which is known to be non-cancerous, is prepared in the same manner. An equal quantity of each of these samples is then mixed, in the presence of oxygen, with firefly lantern extract which includes a mixture comprising luciferin, luciferase and a cation such as magnesium. A reaction will occur which is accompanied by the emission of light. This phenomenon is firefly bioluminescence. The quantity of light is measured and the amount of light emitted by the tissues suspected to contain cancer is compared with the amount of light emitted by the tissue known to be noncancerous. The presence of cancer in the unknown material will be indicated by a significantly less amount of light being emitted by the tissue suspected to contain cancer than the tissue known to be noncancerous on an equal aliquot basis.

The firefly bioluminescent reaction may be carried out utilizing crude firefly lantern extracts or the purified constituents therefrom which participate in the bioluminescent reaction. It has been found that a sufficiently high degree of sensitivity may be attained using the primary extract of the firefly lantern.

Lyophilized firefly lantern extract may be obtained commercially. This material may be prepared for use by dissolving it in distilled, deionized water to the desired concentrations such as, for example, by dissolving 70 mg. of lyophilized firefly lantern extract in 5 ml. of water. The lyophilized preparation will also contain $MgSO_4$ and potassium arsenate in amounts sufficient to result in concentrations of 0.01 M and 0.05 M, respectively. The pH of such a solution is 7.4. The solutions may be further diluted to give any desired concentration of firefly lantern extarct.

The firefly lantern extract which mey be used in the practice of this invention may also be prepared in the laboratory from desiccated firefly tails. The firefly tails are first ground to a fine powder with a mortar and pestle with a small amount of washed silica. The powder is then extracted with 0.05M potassium arsenate-0.01 M $MgSO_4$ at pH 7.4.

When crude firefly lantern extract is used in the practice of this invention, there may be present in the extract small amounts of ATP and ATP precursors, along with phosphorylating enzymes capable of converting the precursors into ATP. The presence of these materials may give rise to a basal light emission by the firefly extract in the absence of exogenous ATP. This type of light emission, which is referred to as inherent light, occasionally may interfere with the detection of light emission in the practice of this invention. However, the problem of inherent light may be eliminated or minimized by one or more of the following techniques:

(1) The firefly extract may be partially purified to remove the factors responsible for the inherent light. The separation and partial purification of luciferase and luciferin is described by McElroy (Methods in Enzymology, vol. II, page 851, Academic Press, Inc., New York, 1955).

(2) Another approach to the removal of inherent light involves the "salting out" of luciferase by the addition of ammonium sulfate to the firefly extract, leaving the non-protein factors responsible for inherent light in the supernatant. This has been accomplished as follows: 50 mg. of lyophilized extract were suspended in 10 ml. of 2.7 M ammonium sulfate. After standing at room temperature for 15 minutes, the suspension was centrifuged

at approximately 200 G for 10 minutes after which the supernatant was discarded. The precipitate, after being washed twice with 10 ml. aliquots of 2.7 M ammonium sulfate, was taken up in 2.5 ml. of a solution of 0.05 M potassium arsenate buffer (pH 7.4) and 0.01 M magnesium sulfate. This treatment, followed by reconstitution with partially purified luciferin, reduces the overall activity of the extract by only about 15% and reduces the inherent light by about 90%.

(3) Experiments have indicated that the use of calcium phosphate gel will also reduce the inherent light in the extract without significantly reducing the activity of the extract. Thus, 50 mg. of commercial lyophilized firefly extract were dissolved in 1.25 ml. of deionized water and centrifuged. The solution was then treated from one to three times with varying amounts of calcium phosphate gel (from 249 mg. to 334 mg.). The treatment consisted of shaking the gel with the extract for ten minutes and then removing the gel by centrifugation at 200 G for 10 minutes. With one treatment with calcium phosphate gel, there is a reduction of about 85% of the inherent light with only a loss of from 7 to 26% of luciferin-luciferase activity.

(4) The simplest means for reducing the inherent light is by dilution of the extract with water. Maximum sensitivity with the least amount of inherent light is obtained at a lyophilized extract concentration of 3 mg./ml.

The practice of this invention may be used to determine rapidly the presence of cancer present in small quantities of tissues. Using electronic equipment, tissues or cells containing an amount of ATP less than 2×10^{-4} μg. and approaching 10^{-7} μg. may be assayed. The bioluminescent reaction itself takes less than 0.5 second to attain maximum amplitude.

Any animal tissue may be tested for cancer by the practice of this invention. The process may be used as a diagnostic tool permitting the rapid determination of a maligancy in clinical or surgical cases. This new method is more rapid than the present biopsy procedures and hence will appreciably decrease the lapse of time required for the diagnosis of patients undergoing surgery and, consequently, the time required for the operation. This invention may also be used in research in conjunction with the detection of virus as described and claimed in concurrently filed application Ser. No. 433,462 filed Feb. 17, 1965, and now abandoned, for "Method for the Detection of Virus." The method may permit a determination of the viral or nonviral origin of specific cancer. The method may also facilitate a study of the kinetics of malignant growth with a view towards elucidating the mechanism and to permit possible control.

In assaying the samples of tissue, results may be obtained by mixing the intact tissue cells with the firefly lantern extract. However, for maximal response and in order to make more accurate assays, it is preferred to comminute the tissue and to rupture the cells and extract the ATP therefrom. A variety of method for the extraction of ATP from the cells may be used. These include hot water extraction, acetone extraction, ultrasonic disruption, dimethylsulfoxide extraction and perchloric acid extraction. Some of the methods which may be used for accomplishing rupture and extraction of the cells are described below:

(A) Acetone and hot water: One ml. of the comminuted tissue is added to 10 ml. of deionized water and the suspension maintained at 100° C. for one to five minutes. The preparation is then cooled and assayed for ATP.

The acetone extraction consists of adding one ml. of comminuted tissue suspension to ten ml. of acetone. After standing for one to five minutes, a one ml. aliquot of the mixture is taken to dryness in air and the residue suspended in one ml. of deionized water. The preparation is then assayed.

(B) Dimethyl sulfoxide (DMSO): DMSO is a high-boiling point organic solvent, miscible with water in all proportions and exhibiting a very low order of toxicity. One ml. aliquots of comminuted tissue suspension are added to ten ml. of various concentrations of DMSO in water. After standing for five minutes, the suspension is assayed for ATP response.

(C) Ultrasonic oscillation: Ultrasonic oscillation has been successfully used by many investigators for the rupture of cells. Five ml. of an aqueous suspension of finely divided tissue are subjected to one to five minutes of ultrasonic oscilaltion at 50 to 75 watts. After cooling the tube in flowing water, the treated suspension is assayed for ATP response. Trichloroacetic acid (0.5 ml. of 5% solution) may be added to the suspension prior to sonification in order to stabilize the ATP against hydrolysis.

(D) Perchloric acid: Up to 0.2 ml. of perchloric acid may be added to 5 ml. of tissue suspension. The preparation is then assayed.

It is preferred to contact the comminuted tissue to be tested and the firefly lantern extract in a liquid reaction medium, such as sterile, deionized water. The liquid reaction medium will generally contain enough dissolved oxygen to allow the bioluminescent reaction to take place.

The material to be assayed for ATP content should be mixed with the firefly lantern extract in a manner which permits the mechanical measurement and recordation of the light emitted. The amount of light emitted is directly proportional to the total ATP content of the material being tested other conditions being equal. Comparisons of normal and malignant tissue are made on the basis of ATP content per unit tissue volume or weight or per unit protein content, or any combination thereof.

The procedure for using the instruments which are used to detect and record the intensity of emitted light consists of injecting a liquid medium containing the material to be assayed, such as an aqueous extract of the material, into a cuvette containing the firefly latern extract. The extract is held at pH 7.4 with potassium arsenate buffer. The light emitted as the result of the reaction between the ATP in the tissue culture to be tested and the firefly lantern extract strikes the surface of a photomultiplier tube giving rise to a current which can be measured and recorded by either an oscilloscope photograph or a linear recorder. The unit of intensity used for comparing these reactions is the millivolt. The unit of light intensity has been arbitrarily defined as being equivalent to one millivolt. Alternately, a pulse counting device with a digital or analogue read-out may be used to record the reaction.

Because the response (i.e., light emission) is almost instantaneous when the tissue culture is contacted with the firefly lantern extract, the extract should be positioned in front of the light detection system prior to the introduction of the material to be assayed.

There are two ways in which the bioluminescent response with ATP present in a material can be expressed. One is by measurement of the maximum intensity of the emitted light, which after reaching this maximum value, decays exponentially. With all other factors constant, the maximum intensity is directly proportional to the concentration of ATP. The alternative manner of expressing the response is by integration of the total amount of light emitted; i.e., area under the light intensity curve. This is the slower of the two methods, because of the relatively long time necessary for complete decay (up to 10 minutes). Therefore, maximum intensity has been chosen as the measure of ATP response.

The instrumentation necessary for the quantitative measurement of bioluminescence consists of a photomultiplier tube for the conversion of light energy into an electrical signal, a device for determining the magnitude of the signal, and a light tight chamber for presentation of the bioluminescent reaction to the photomultiplier tube.

In one system, part of the assembly consists of a composite sensing and reaction chamber which contains a photomultiplier tube, with appropriate circuitry, and a rotary cylinder mounted in a block of aluminum in a man-

ner which permits removal of the reaction chamber without exposing the phototube to light. A section of the cylinder wall is cut out to accommodate a standard ten mm. or five mm. rectangular cuvette. Immediately above the cuvette holder is a small injection port sealed with a replaceable light-tight rubber plug. The entire unit is painted black to reduce light reflection. The photomultiplier converts the light energy into an electrical signal. An oscilloscope, which records the magnitude of the signal from the photomultiplier, is provided with a maximum sensitivity of 200 μv./cm. of beam deflection which will allow an increase in system sensitivity by decreasing the bandwidth or directly reducing the noise level. There is a multiple switching arrangement at the scope input which makes it convenient to adjust the system zeros and balances. The differential input to the scope provides a means to balance the dark current output of the phototube. The response to the firefly luminescent system displayed on the oscilloscope screen is recorded with a camera which mounts directly onto the front of the oscilloscope. To observe and record the reaction, the cuvette containing the necessary reagents is positioned in the cuvette carrier without exposing the phototube. Rotation of the carrier positions the cuvette in front of the phototube. The unknown, is then added through the injection port and the magnitude of the response, if any, is recorded by the camera.

The overall sensitivity and perhaps reliability of the bioluminescent reaction of the material to be tested may be increased by the conversion of other nucleotide phosphates which are present in tissues, such as adenosine diphosphate (ADP) and adenosine monophosphate (AMP), to ATP. This may be accomplished as described below by adding to the tissue culture certain phosphorylating enzymes. One such enzyme is phosphocreatine kinase.

Phosphocreatine kinase (10–30 units/mg.) is prepared in a concentration of 0.4 mg./ml. in 0.05 M potassium arsenate buffer (pH 7.4) containing $MgSO_4$ at concentration of 10^{-5} M and creatine phosphate at a concentration of 0.1 mg./ml. One tenth ml. of this solution is added to 1 ml. of a solution being assayed for ADP. The mixture is allowed to incubate at 30° C. for 30 minutes at room temperature and is then boiled for five minutes to destroy the kinase. After cooling at 0° C., a $\frac{1}{10}$ ml. aliquot is removed for ATP assay. The difference between the ATP content prior to incubation with phosphocreatine kinase and the ATP content after such incubation is the amount of ADP which has been converted to ATP.

The following examples illustrate the practice of the invention.

EXAMPLES 1 TO 4

In these examples, different tissues are obtained in both cancerous and noncancerous form. The noncancerous tissues are taken from the same animals and organs as those containing the malignancy and are used as controls. The cancerous tissues contain the type of tumorous growth listed in Table I.

A quantity of commercially obtained lyophilized firefly lantern extract corresponding to 50 mg. of firefly tails is dissolved in ten ml. of distilled water. After centrifugation at 1700 x G to remove debris, the solution, which contains luciferase, luciferin, $MgSO_4$ -10^{-3} M, and arsenate buffer -10^{-2} M, is ready for use. One ml. of the extract is placed into a cuvette which is then positioned in the light detection chamber of an apparatus as previously described. Sufficient dissolved oxygen for the bioluminescent reaction is present in the extract.

Before the tissues containing unknown concentrations of ATP are assayed, a calibration of known concentrations of ATP is plotted by injecting $\frac{1}{10}$ ml. portions of known concentrations of ATP through the light proof seal into the cuvette by means of a hypodermic syringe. The light response in millivolts is plotted against the ATP concentration. A straight linear function is obtained. For example, the response from 10^{-1} gamma of ATP is 20,000 millivolts while that from 10^{-2} is 2000 millivolts, etc.

A sample (500 mg.) of each tissue is minced with a scalpel and suspended in a solution composed of 4.5 ml. of distilled water and 0.5 ml. of 2% (by weight) trichloroacetic acid. The suspension is boiled for 5 minutes and then cooled to approximately 0° C. in an ice bath. Each sample is then brought up to a volume of 6 ml. with distilled water after which it is subjected to ultrasonic rupture at approximately 75 watts power for one to five minutes. The samples are then cooled to approximately 0° C., in an ice bath. One ml. of each sample is diluted to a final volume of 10 ml. with distilled water. One tenth ml. aliquots of each sample is assayed for ATP content using the firefly bioluminescence technique by injecting the aliquot into the cuvette of the apparatus and measuring the amount of light emitted. The results are set forth in Table I. The ATP content, in this example, is expressed in terms of micrograms of ATP per milligram of wet tissue cells.

TABLE I

	Type of tumor	APT Content
Example:		
1	Carcinoma	0.008
	(Control)	0.115
2	Heptoma	0.021
	(Control)	0.125
3	Sarcoma	0.054
	(Control)	0.089

It is seen that the ATP content of the cancerous tissues is considerably lower than the corresponding controls—i.e., non-cancerous tissues.

EXAMPLES 5 TO 9

The mouse and monkey tissues listed in II which are suspected to be cancerous are obtained. Corresponding tissues which are known to be noncancerous are obtained from the same animal and organs as the suspected malignant tissues. The tissues are all assayed for ATP content by the process described in Examples 1 to 4. In each example, the ATP content of the tissue suspected to be cancerous is significantly lower than the corresponding non-cancerous tissue, ranging from 10 to 30% of that present in equal aliquots of the non-cancerous tissue. This confirms the suspected presence of cancer in the cells tested.

TABLE II

Example:	Tissue
5	Erythrocytes (mouse).
6	Leucocytes (mouse).
7	Axillary region (mouse).
8	Liver, kidney (mouse).
9	Kidney (monkey).

We claim:

1. A method for determining the presumptive presence of cancer in a tissue which comprises preparing a sample of the tissue suspected to contain cancer, preparing an equal quantity of a sample of adjacent tissue from the same animal and organ which is known to be noncancerous, mixing each of said tissues in the presence of oxygen with firefly lantern extract, said extract containing luciferin, luciferase and magnesium ions, measuring the amount of light emitted by the reaction of each of said samples with said extract, comparing the amount of light emitted by said sample suspected to contain cancer with the amount of light emitted by said sample which is known to be noncancerous, the presence of cancer being indicated by a significantly lesser amount of light being emitted by said sample suspected to contain cancer than said tissue known to be noncancerous.

2. A method for determining the presumptive presence of cancer in an animal tissue which comprises obtaining a sample of the tissue suspected to contain cancer

and another sample of tissue from the same animal and organ which is known to be noncancerous, comminuting each of said samples, preparing an aqueous suspension of equal amounts of each of said comminuted samples, treating each of said suspensions to rupture the cells of said tissues and extracting the adenosine triphosphate therefrom, thereafter mixing said treated suspensions of sample in the presence of oxygen with firefly lantern extract, said extract containing luciferin, luciferase and magnesium ions measuring the amount of light emitted by the reaction of each of said suspensions of sample with said extract, comparing the amount of light emitted by said suspension of sample suspected to contain cancer with the amount of light emitted by said suspension of sample which is known to be noncancerous, the presence of cancer being indicated by a significantly less amount of light being emitted by said suspension of sample suspected

to contain cancer than said suspension of tissue known to be noncancerous.

3. The method of claim 2 wherei said suspensions are subjected to ultrasonic oscillation in order to rupture the cells of said tissues.

4. The method according to claim 2 wherein the amount of light emitted is increased by the addition of phosphocreatine kinase.

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