5-7-1974

Oxygen Consumption by Acrylamide Polymerization for Rapid Screening of Chemotherapeutic Agents

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OXYGEN CONSUMPTION BY ACRYLAMIDE POLYMERIZATION FOR RAPID SCREENING OF CHEMOTHERAPEUTIC AGENTS

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Ever since the advent of chemotherapy in the treatment of cancer there has been a need for a rapid, sensitive screening technique for establishing the possible effects of the various anti-cancer drugs on specific tumors.[1] Various screening methods have been established which base their conclusions on the histological effects of the drugs on the tumor. These methods are based on (1) measurements of size decrease of solid tumors, (2) on histological changes in tissue cultures of the various tumor cells and (3) on decreases in uptake of radioactive precursors of drug treated cells.[2,3,4,5,6,7] Other methods used are based on the measurement of the production of various molecules such as total protein, nucleic acid, and lactic acid.[8,9,10] All of these techniques are involved, requiring highly skilled technical personnel and expensive equipment as well as from 1-30 days for completion of results.

We have developed an alternative to these systems. Our screening process can determine the overall effect of all possible anti-cancer drugs and combinations thereof at various concentrations on a specific tumor within 3 hours after removal of a biopsy on the morning of surgery. We do this by suspending the tumor cells in Medium 199, and during incubation at 37°C., measuring the rate of oxygen consumption of a drug treated and control group of cells. This screen is based on the fact that in respiring cells of the human body, oxygen consumption is a prime indicator of a cell's metabolic state

and/or potential viability.[11,12,13] This fact, in turn, is based on the simple idea that aerobic cells respire, and therefore, consume oxygen if they are viable, and on tumor angiogenesis studies. Tumor angiogenesis is the phenomenon of blood vessel growth to or from tumors as the tumor size increases, thus alleviating the mass' need for more nutrients and oxygen.[14] As the tumor size increases there occurs a need for more oxygen and nutrients which are supplied by the increased blood vessel growth. If this blood vessel supply is inhibited or decreased then the tumor will either stay the same size or shrink due to its lack of nutrients and adequate oxygen. The tumor cells could therefore be exposed to various chemotherapeutic agents to see how these alter a cell's oxidative metabolic potential. A decrease in oxygen consumption is therefore an indicator of cellular metabolic insufficiency in those treated cases such as ours where the cells are adequately perfused with nutrients and oxygen and some chemical damage has been done to the cells molecular mechanisms by an anti-cancer drug. Because our technique measures a quantitative parameter we are also able to show which drugs have the greatest effect and which the least; or, in other words, the degree to which one drug is effective relative to another.

Oxygen content readings of control and treated groups of cells over a 3-4 hour period allow for construction of characteristic O_2-consumption curves. We measured oxygen content by a technique originally developed in Dr. Folkman's lab for the measure of O_2 content in blood. This technique will be explained later.

Using three common anti-cancer drugs we measured the oxygen consumption of Walker 256 ascites tumor cell suspensions. With Actinomycin-D,

an RNA- synthesis inhibitor, the control group consumed O_2 at the rate of 6.8µl./10^6 cells/hour while the same cells treated with 0.13µg/ml. Dactinomycin respired at a rate of only 1.4µl./10^6 cells/hour reflecting an 80% inhibition in the suepension's cellular oxidative metabolism. With Cycloheximide, an inhibitor of protein synthesis, the control group respired at 9.0µl./10^6 cells/hour while the drug treated group consumed O_2 at only 3.2µl./10^6 cells/hour, a 65% inhibition. With Cytosine Arabinoside, an inhibitor of DNA synthesis, the control group of cells consumed 23µl.O_2/10^6 cells/hour while the drug treated cells respired at only 8.7µl.O_2/10^6cells/hour, an inhibition of 62%. These figures represent the mean values of all the control and drug-treated groups within each drug group tested. They are presented in this way to give an overall picture of what has been accomplished. It must be noted however that each test of a control and treated group must be taken individually on any single day. The reason for this is clear. Each tumor has its own characteristic metabolic rate which is effected by a multitude of variables, the most important being the reaction or lack of reaction of the host to the tumor. This will be true also in those tumors biopsied from individual patients in the clinic. Each patient, and therefore each tumor, may have a unique metabolism with regard to rate end response of the host to the tumor. There may also be such a differential reeponse process as far as individual tumor- drug relationships are concerned. This screen is not meant to be treated statistically but is a clinical tool for assessing each individual tumor for specific chemotherapeutic regimens. This can be illustrated nicely by refering to some of our

experiences in the collection of data. Although we were utilizing the same cell line- Walker 256 ascites tumor- the calls were grown in rats which, although belonging to the same species, had individual metabolic rates and responses of their own which in turn effected the invading tumor cells. Collecting these tumor cells everyday from 20 distinct rats and pooling them into one suspension had a likewise variable effect on the net metabolic rate in our suspension. This is dynamically illustrated by our various results. Another illustration of this metabolic 'rate-response' or unique tumor phenomenon occured over Christmas when we grew our cells in solid phase as we were away. Upon returning we found our oxygen-consumption rates to be 20-30 times what they were before Christmas. This happened as a result of a selection process in which those cells -the most active- which grew best in solid phase proliferated and in effect resulted in a population of metabolically different solid phase cells.

The percent inhibition of oxygen consumption thusly obtained proved to correlate well with the percent size shrinkage of solid tumors as determined by the National Cancer Institute's Tumor Study Program. According to Emil Frei, the program's director, the NCI screened various antineoplastic agents by noting the size reduction of Walker 256 solid tumors after treatment with the drug. This size shrinkage would also be accompanied by a proportionate decrease in the net oxygen consumption of the mass of cells. Following is a comparison of our figures with those obtained by the NCI study:

DRUG	% O_2 Consumption Inhibition	% Size Decrease
Actinomycin D	80%	82%
Cycloheximide	65%	60%
Cytosine Arabinoside	62%	70%

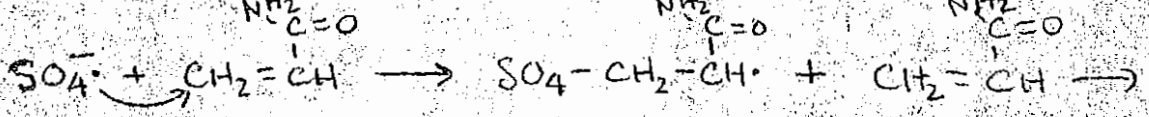
This is a potentially powerful tool for the cancer chemotherapist. The reason for its rapidity relative to the other methods is simple. In measuring histological changes in tissues or size decrease of tumor masses one is measuring the effect of an altered metabolism and the visible effects of these changes occur long after the initial gearing down or damaging of the molecular mechanisms and energy chain. In measuring radioactive uptake it is necessary to spend much time in preparation of your sample and even though you may be able to measure a portion of the metabolic effect by using certain radioactive precursors you are unable to see the total picture of the cell's condition since you are looking at only a handful of the molecular contents. Since oxygen is an indicator of the overall cellular condition due to its linkage to all energy requiring processes in the respiring cell it is an ideal parameter to watch. And since oxygen consumption measures are able to "show" the initial actual gearing down or breaking down process of the metabolic mechaniems due to certain drugs it provides a much more rapid and sensitive pre-measure of the later occuring tissue effects which we can se with the aid of the microscope.

METHOD OF OXIMETRY

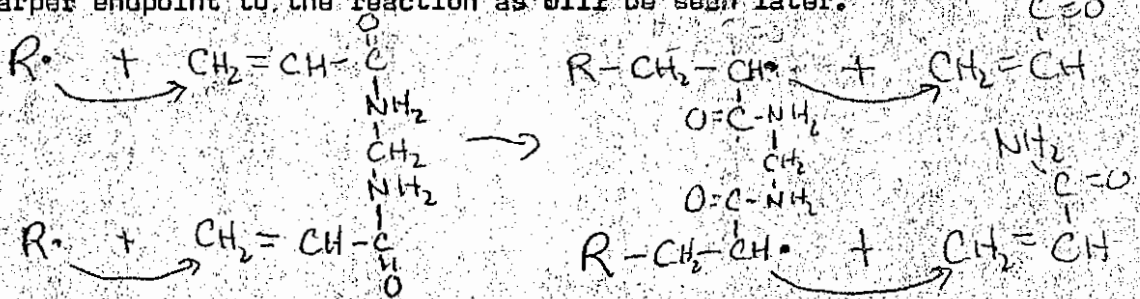
Our method for measuring oxygen content is based on a technique developed in Dr. Folkman's Lab for the determination of the oxygen content in blood.[12] The technique grew out of observations made by Dr. Folkman in preparing acrylamide gels for the separation of Tumor Angiogenesis Factor. It is based on the well known fact that oxygen inhibits free-radical polymerization. A solution of acrylamide and bisacrylamide are

are polymerized and the amount of oxygen present is directly proportional to the logarithm of the polymerization time. This is actually the gelation time, but for all intensive purposes we may call it the APT or Approximate Polymerization Time. Here is a quick scheme of what occurs in the reaction chamber of our apparatus:

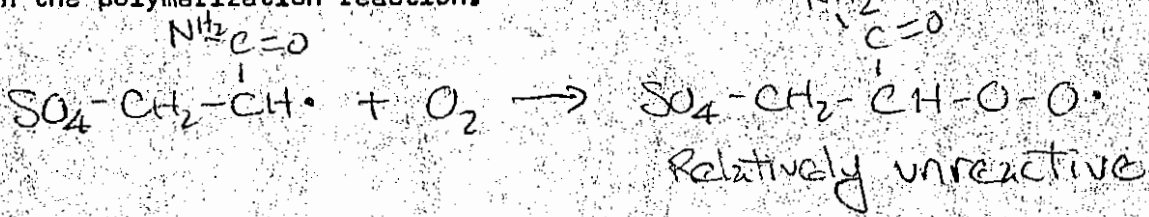
1. INITIATION - The polymerization is commenced by sulfate radical ions $-(SO_4^{\cdot -})-$ generated by the scission of a weak peroxide bond in persulfate ions $-(S_2O_8^{=})-$. A reduction activator -bisulfite (HSO_3^-)- provides the activation energy advantage for this scission. The sulfate radical ion attacks the double bond of an acrylamide molecule, forming a radical intermediate which propagates the chain reaction and forms a progressively longer chain until inhibition or chain termination.



N,N'-methylenebisacrylamide permits cross linking between polymer chains which helps to make a more solid gel upon gelation, a property which gives a sharper endpoint to the reaction as will be seen later.



2. INHIBITION - Acrylamide radicals react with oxygen preferably to form a relatively stable and unreactive intermediate that ties up polymer chains and creates a lag period while oxygen is being consumed in the polymerization reaction.



After all the oxygen has been used, the polymerization proceeds to gelation and completion. We therefore will observe a longer APT with increasing amounts of oxygen in the eample.

The reaction vessel consists of a ten cc. syringe capped with a rubber dummy and in which is contained a small one-half inch stirring bar. 1.2 cc. of a buffered solution of acrylamide and bisacrylamide are introduced into the ten cc. syringe with a 3 cc. syringe. After injecting the solution the same 3 cc. syringe is used to withdraw all of the excess gas within the reaction chamber. The reaction chamber is then placed in the reaction cylinder around which is wrapped an electrical hookup wire. This entire apparatus is situated over a magnetic stirrer which causes the bar magnet within the reaction chamber to spin, thus mixing the contents as furthur solutions are added. .6 cc. of the ammonium persulfate is added next through the rubber dummy injection portal. This is followed by the injection of .3 cc. of sample - in our case this would be .3 cc. of cell suspension. Waiting thirty seconds for thorough mixing, .9 cc. of the sodium bisulfite solution is added and the reaction begins. At the same time a digital timing device is started by pushing a button linking the timing circuit with that of the electrical hookup wire. The counter continues to count as the small rotating bar magnet induces a small alternating current within the hookup wire due to electromagnetic induction. As soon as the reaction chamber contant has been completely polymerized the stirring bar can no longer turn, thus reducing the output voltage from the coil and stopping the digital readout at the APT.

EXPERIMENTAL DESIGN

Our experimental design is very simple, but it took a long period of trial and error to develop the ideal system. We would harvest the tumor cells and suspend them in Medium 199. We harvested the Walker 256 ascites tumor cells from 20 rats on each of 50 days which were then incubated for 1-3 hours and the APT taken hourly. Fifteen cc. of each daily suspension was introduced into each of four small spinner flasks and appropriate flasks were treated with drug. The spinners were then sealed and samples taken anaerobically through the side arm injection portal for testing at time 0. During the incubation at 37°C. a sample was tested every hour. The data was collected in this way and interpreted in the following manner.

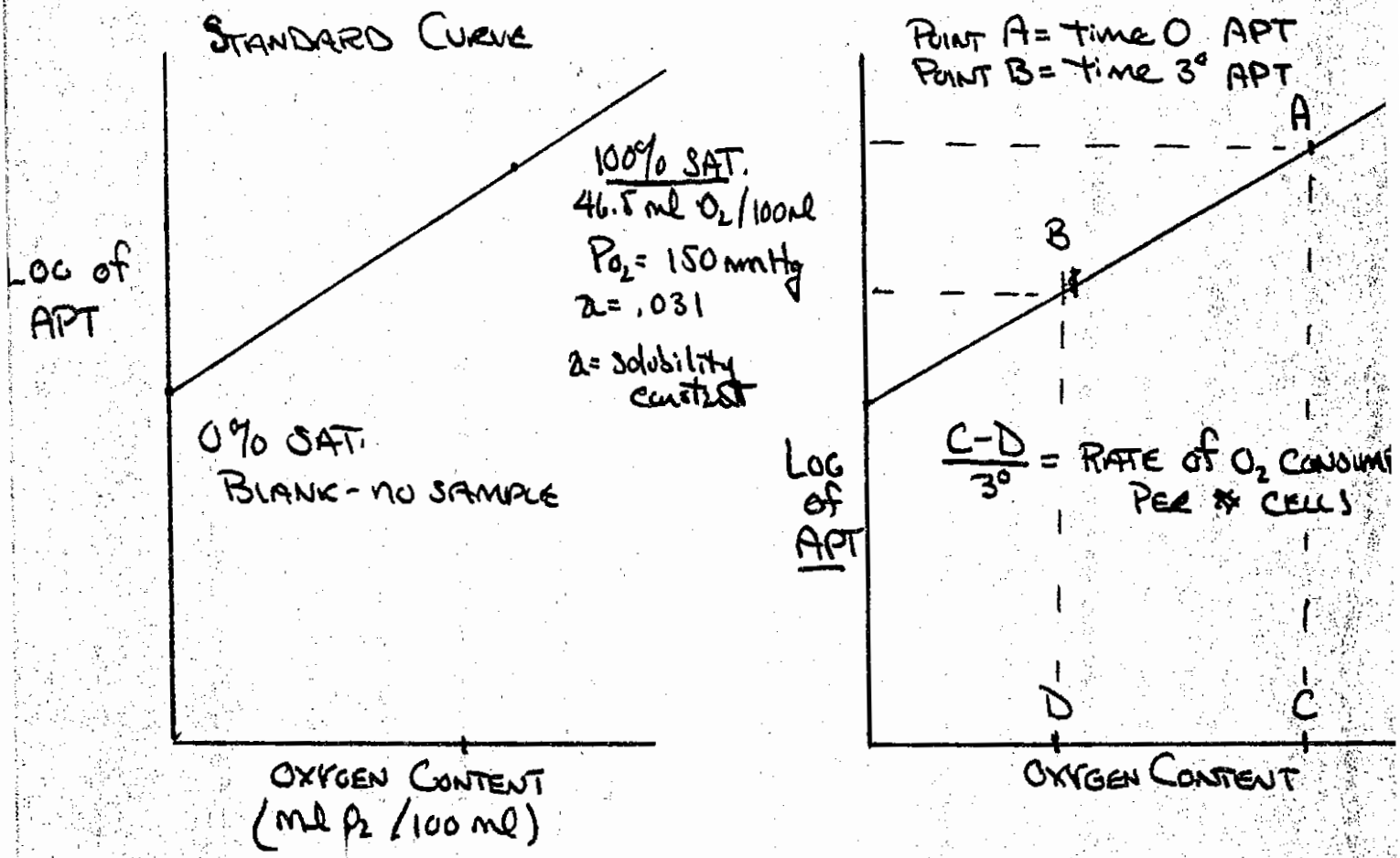
DATA INTERPRETATION

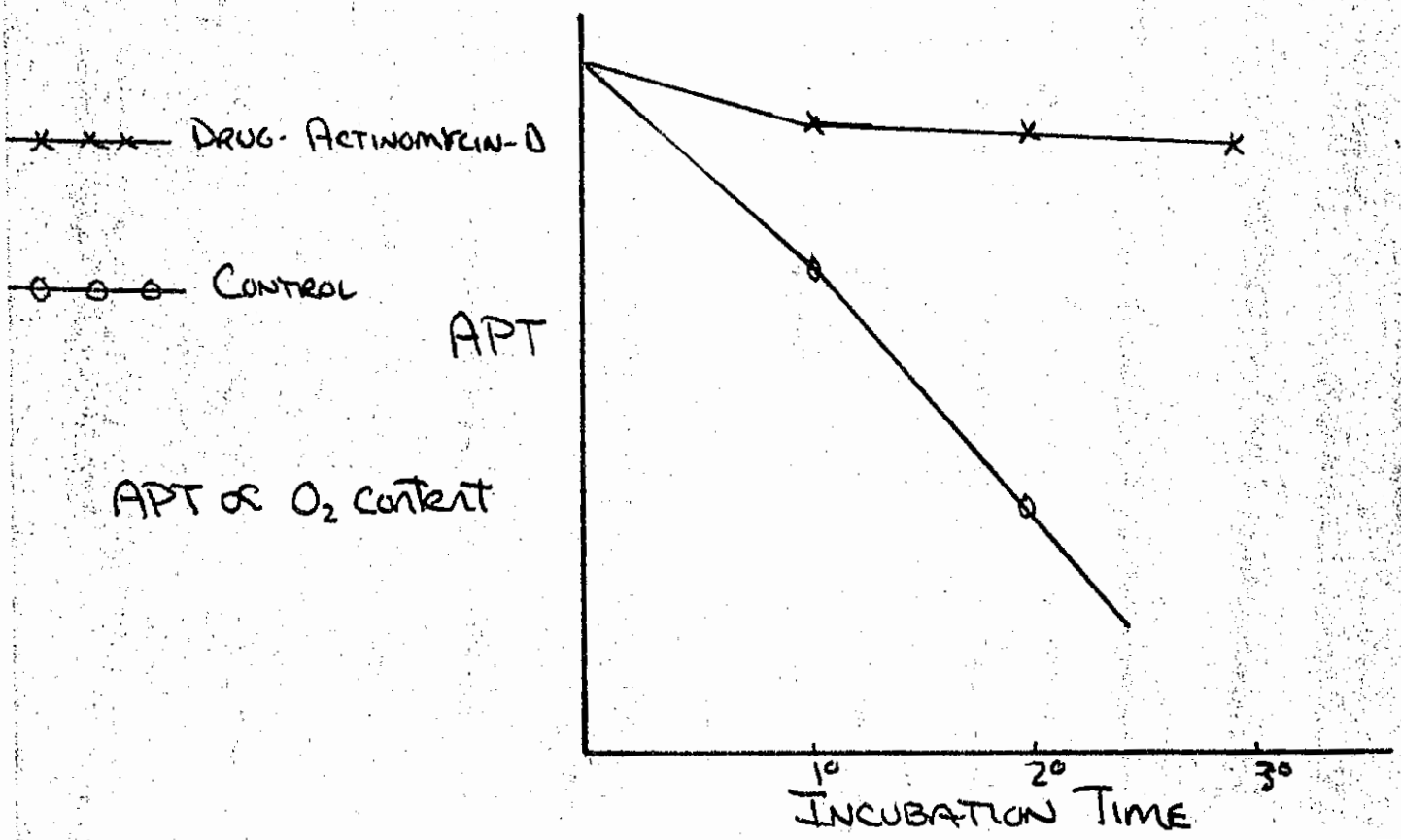
Po_2 values for the medium were obtained every day on a Radiometer Blood-Gas Analyzer and converted to oxygen content by multiplying by the solubility constant of the medium. The solubility conetant in turn had been determined by utilizing Van Slyke Apparatus in conjunction with the Radiometer. The mean oxygen content of the medium was found to be 46.5 ml O_2/100 ml medium in this way. At the same time the APT of the medium was obtained and these to figures constituted the upper point or 100% saturation point of our standard daily plot of APT vs. O_2 content of the suspension. As the cell suspension became more dilute the APT and O_2 content values approached those of the medium, the difference between them being negligible. Therfore, this curve was almost identical to that for the suspension and was the one used in comparing the treated and control group figures for oxygen consumption. The 0% saturation

point was taken to be at 0 oxygen content in ml O_2/100ml medium and at that APT which characterized the polymerization time of the reacting solutions alone - the blank. A straight line was drawn between the two points and a standard curve constructed. Each APT value could thus be interpreted as a specific oxygen content. This relationship was tested utilizing the Radiometer on each sample tested to make certain our suspension values did indeed fall along our standard curve - and they did! This verified the same results obtained when studying blood - that the logarithm of the APT was directly proportional to the oxygen content of the medium. Utilizing this standard curve we were able to convert our APT's directly into oxygen content - and setting this up on a time curve we were able to establish the O_2 consumption rates of the tumor cell suspensions. The utilization of another method of oximetry was found to be unnecessary since our 100% saturation level remained essentially constant at 46.5 ml O_2/100ml, and therefore our daily standard curve could be constructed with only the APT's of the medium and the blank.

The oxygen consumption rates were established as follows. The difference between any two APT readings was converted into a corresponding content of utilized oxygen by using the standard curve constructed on the same day. Dividing this figure by the time elapsed of incubation period gave us the oxygen consumption in terms of ml O_2 per hour per number of cells in the suspension which was determined by a haemocytometer. This in turn was converted into liters (µl) of oxygen consumed per hour per 10^6 cells. A comparison of the control rate with the drug treated rate would determine whether or not the drug was effective and just how effective it was in comparison with other drugs.

A plot can be made of the APT's vs. incubation time and a visual representation of the drug's effect can be made.





ERROR ANALYSIS

Our ablility to measure oxygen content precisely by this method is adequately ilustrated by our ability to obtain repetative results with the blank and medium APT's. Our mean standard deviation for obtaining these APT's was ±.4 seconds. The standard deviation of oxygen content values found by this method on graphs was ± 1.1µl/10^6 cells. This is comparable to any technique of oxygen consumption measure existing today.

CONCLUSION

It should be noted that scientists have had this ability and knowledge to measure oxygen consumption for some time now. What we have done is merely apply this ability and knowledge to a present day problem - that of determining ideal chemotherapeutic programs quickly in the treatment of a cancer. We not only found that this application worked but we also developed a simpler, less expensive method of measuring the oxygen consumption phenomenon upon which we based our experimental design.

SOME REPRESENTATIVE OXYGEN CONSUMPTION VALUES in $\mu l/10^6$ cells/hour

Drug	Control	Treated
Actinomycin D	4.2	1.7
	.9	.8
	10.8	.7
	11.5	2.7
Cycloheximide	22.0	8.0
	3.4	2.9
	4.2	1.0
	6.5	1.2
Cytosine Arabinoside	6.1	.4
	40.0	17.0

NOTES

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