United States Patent [19]

Folkman

[11] **3,864,084**

[45] **Feb. 4, 1975**

[54] **METHOD FOR MEASUREMENT OF OXYGEN CONTENT IN BLOOD AND OTHER LIQUIDS BY OXYGEN INHIBITION OF CHEMICAL REACTIONS**

[76] Inventor: **M. Judah Folkman,** 18 Chatham Cir., Brookline, Mass. 02146

[22] Filed: **Aug. 3, 1972**

[21] Appl. No.: **277,810**

[52] **U.S. Cl.** ..... **23/230 R,** 260/89.7 R, 23/230 B

[51] **Int. Cl.** **C08f 3/90,** G01n 33/16

[58] **Field of Search** ..... 23/230 B, 230 R; 260/89.7 R

[56] **References Cited**

UNITED STATES PATENTS

1,513,542	10/1924	Flagg	23/230 B X
3,073,808	1/1963	Mertz	260/89.7 R X
3,572,995	3/1971	Martin et al.	23/230 B
3,573,263	3/1971	Gill	260/89.7 R
3,583,960	6/1971	Ishii et al.	260/89.7 R X
3,663,518	5/1972	Patzelt	260/89.7 R X

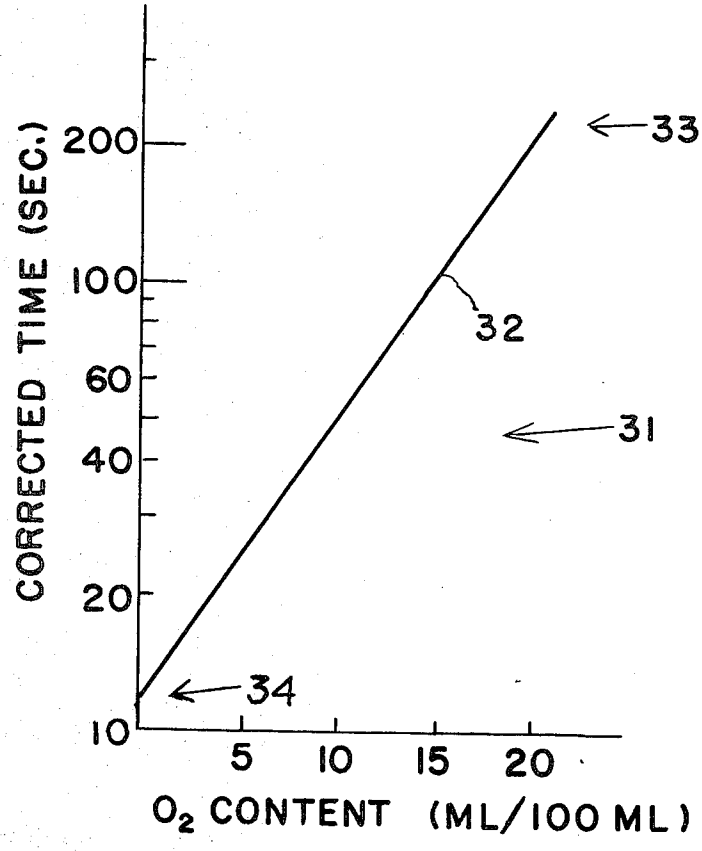
Primary Examiner—Robert M. Reese

Attorney, Agent, or Firm—John E. Toupal

[57] **ABSTRACT**

Method for measuring the oxygen content of blood. The blood sample to be analyzed is mixed with a monomer solution and free radical initiated polymerization is induced and timed. Oxygen in the sample inhibits polymerization. The logarithm of the time required to polymerize is linearly proportional to the oxygen content of the sample. Liquids other than blood can be analyzed by the disclosed method.

25 Claims, 5 Drawing Figures



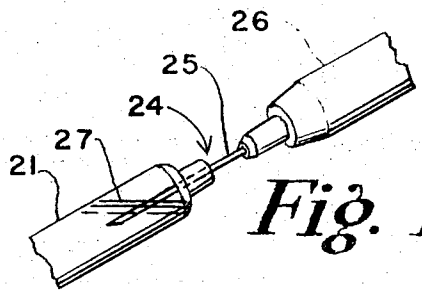


Fig. 1.

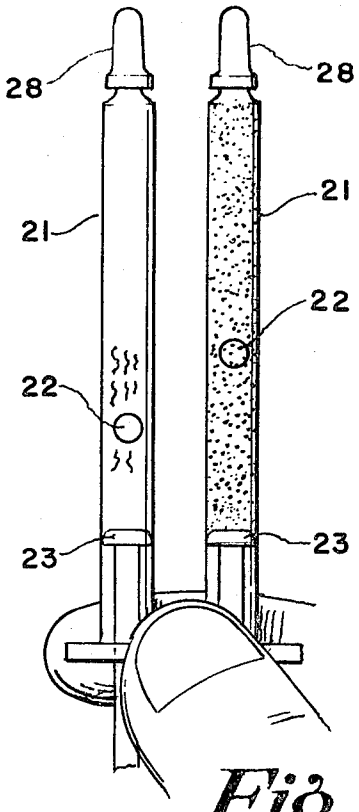


Fig. 2.

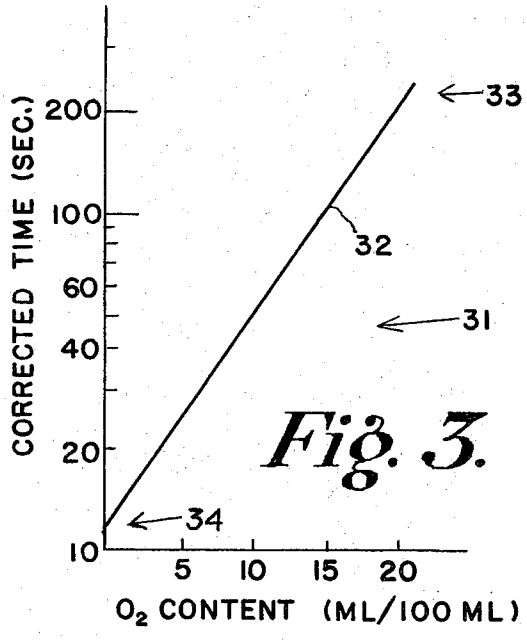


Fig. 3.

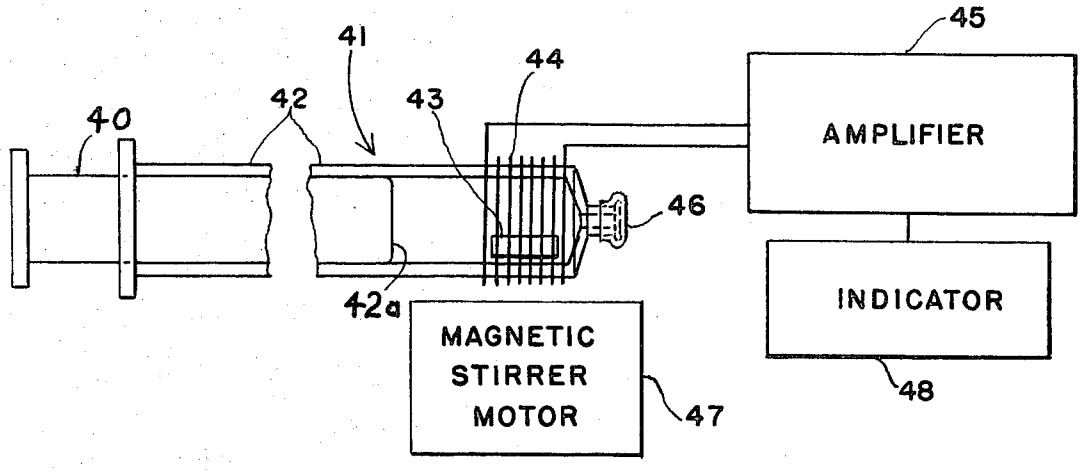


Fig. 4.

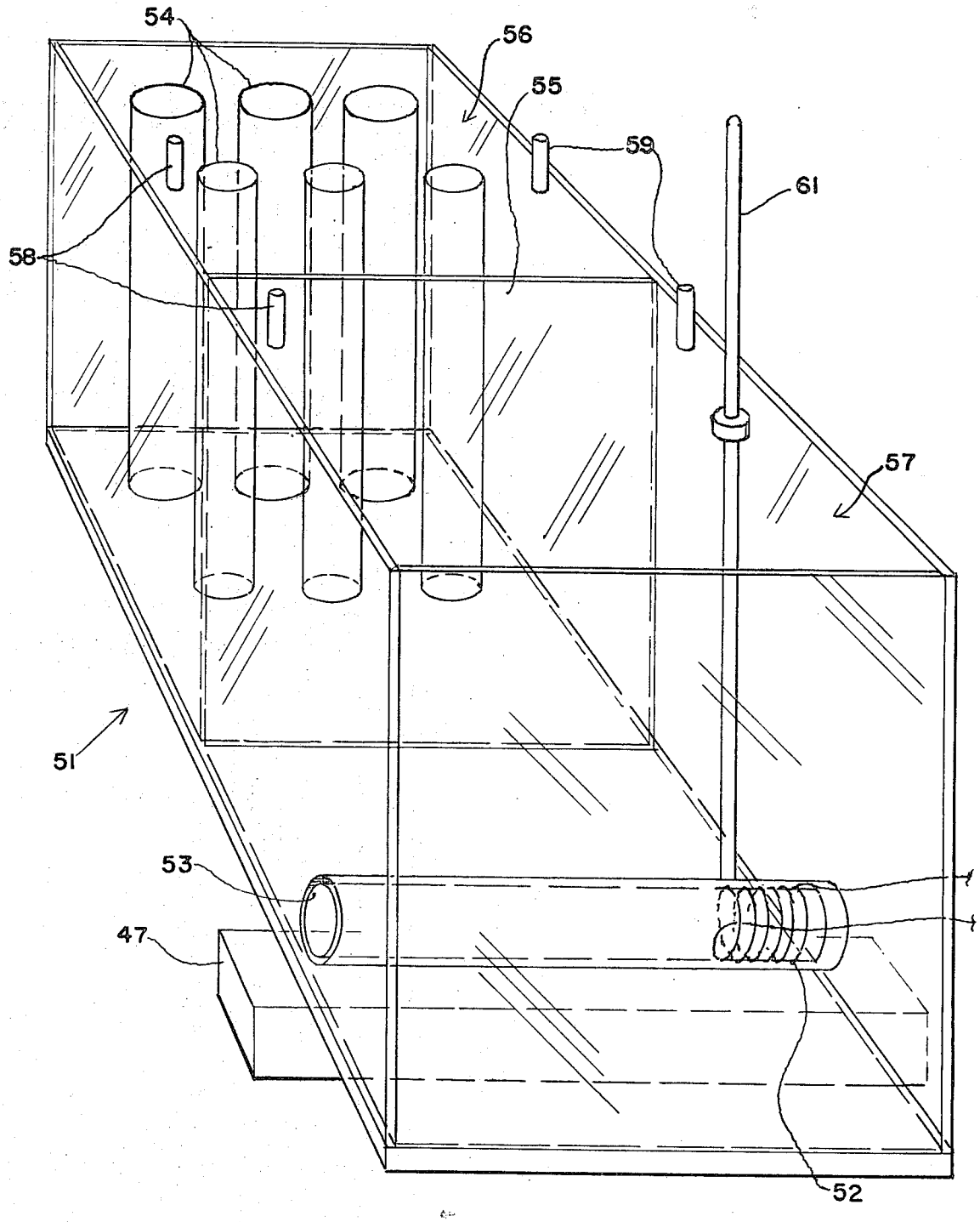


Fig. 5.

METHOD FOR MEASUREMENT OF OXYGEN CONTENT IN BLOOD AND OTHER LIQUIDS BY OXYGEN INHIBITION OF CHEMICAL REACTIONS

BACKGROUND OF THE INVENTION

This invention relates to a method for measuring the oxygen content in blood and other liquids and, more particularly, to measurement by timing inhibition of free radical initiated polymerization caused by oxygen.

Van Slyke and Neill introduced the manometric method for oxygen analysis of blood which has remained the standard against which other methods are judged. In essence, it involves liberation of oxygen from a blood sample by a combination of hemolysis, conversion of hemoglobin to methemoglobin, and vacuum extraction; measurement of the pressure exerted by the liberated gas at a fixed volume; then absorption of the oxygen with a reducing solution and a second measurement of pressure. Since the volume is fixed and the temperature can be measured, the pressure difference gives the oxygen content by the ideal gas law.

This method is cumbersome, with several important disadvantages. First, each determination is time-consuming, requiring about 15 minutes for completion. Second, the manipulations required are numerous and exacting; there are many openings for error and a great deal of training and experience is necessary if a technician is to master the technique. Third, the ordinary sample size is large, usually 1.0 ml, a significant problem in repetitive followup studies in very small pediatric patients. Fourth, dissolved anesthetic gases such as N_2O adversely affect the results obtainable because of incomplete removal by the reducing solution.

Gas chromatography has been applied to the problem. In this method, the gas peak corresponding to oxygen is integrated and related to the total gas volume and sample volume. The method is said to be accurate and reproducible, but requires a large outlay of money for the instrument and sophisticated training for its operator.

With the invention of the Clark oxygen electrode came the possibility of polarographic methods. The Clark electrode gives a rapid direct reading of oxygen tension (pO_2) on the basis of current developed at fixed voltage between a platinum electrode exposed to the oxygen-containing solution through an oxygen-permeable membrane, and a reference electrode. Oxygen tension can be used to calculate oxygen saturation from a standard oxyhemoglobin dissociation curve. The oxygen content can be derived if the oxygen capacity of the blood in question is known (on the basis of hemoglobin level: each gram of hemoglobin binds a constant 1.34 ml of oxygen). This method is adaptable to very small sample volumes and the measurement can be made rapidly; however, there are several major drawbacks. As an indirect method, it is vulnerable to inaccuracies related to the intermediate steps in calculation. The standard dissociation curve itself, for example, is now thought to shift position in consonance with levels of 2,3-DPG and possibly other factors, in response to hypoxia, anemia, stress, etc. The values of factors which affect the curve, especially temperature and pH, must be known accurately. Hemoglobin must be determined independently. The number of ancillary values which must be obtained makes this method somewhat complicated and open to error.

A second polarographic approach has been to measure the increase in pO_2 resulting from liberation of bound oxygen from hemoglobin in dilute solution when the hemoglobin is oxidized to methemoglobin, and from this increase calculate the oxygen content. In skilled hands this method is accurate and requires only microliter samples. However, the apparatus is expensive and sophisticated.

The object of this invention therefore is to provide a rapid method of measuring oxygen content of blood that does not require highly trained technicians or elabrate equipment, yet will provide accurate, reproducible results.

SUMMARY OF THE INVENTION

This invention is characterized by a method for measuring the oxygen content of liquids. A test liquid, containing oxygen, is mixed with an activator liquid and reacts therewith to produce a product at a rate dependent on the quantity of oxygen present. The rate at which the product is produced is timed and thus provides an indication of the quantity of oxygen in the test liquid. As described more fully below, the reagents should be isolated from any gaseous phase and temperatures should be regulated. In addition, depending on the process and the accuracy desired, the reagents may be degassed before use as is discussed below. Among the advantages of this method is the small volume of test liquid required. In addition, results are accurate and repeatable and up to 20 tests per hour can be made. Furthermore, pH of the test liquid does not affect the result if the reagents are properly buffered. If the test liquid includes blood the results are unaffected by pCO_2 and drugs commonly given to postoperative cardiac patients.

The timing step includes monitoring the viscosity of the product and comparing the measure of time obtained to a standard of comparison such as a chart or a graph. For example, disclosed herein is a reagent system utilizing the oxygen inhibition of polymerization of vinyl compounds. In this system, when polymerization is complete, a gel is formed thus producing a radical difference in viscosity and a clearly defined end point. Therefore, comparison of the measured time quickly and accurately provides a measure of the oxygen in the test liquid.

Although the subject method was intended to aid in the oximetry of blood it has been found useful for other liquids such as fluorocarbons and water. Thus, other uses for the method become evident. For example, if a fluorocarbon is permitted to equilibrate with a gaseous phase and oximetry is then performed on the fluorocarbon in accord with the disclosed method, measure of the oxygen in the gaseous phase is supplied.

DESCRIPTION OF THE DRAWINGS

These and other features and objects of the present invention will become more apparent upon a perusal of the following description taken in conjunction with the accompanying drawings wherein:

FIG. 1 shows a syringe being filled with reagent by a hypodermic needle in accordance with the subject method;

FIG. 2 shows 2 syringes filled with the reagents and each containing a glass bead wherein the reaction in the left syringe is incomplete but the reaction in the

right syringe has reached the point of gelation and the glass bead therein has become immobilized;

FIG. 3 is a graph of reaction time v. oxygen content that is a typical standard of comparison;

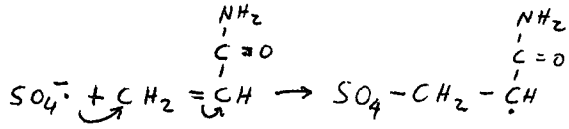
FIG. 4 shows schematically apparatus for practicing the subject method semiautomatically with electromagnetic monitoring apparatus; and

FIG. 5 is an isometric view of a water bath apparatus utilized in conjunction with the syringe mixing chamber shown in FIG. 4 to practice the subject method under controlled temperature conditions.

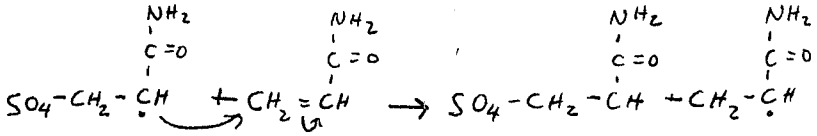
DESCRIPTION OF THE PREFERRED METHOD

The rate at which many chemical reactions proceed is affected markedly by the presence or absence of oxygen. Thus many reactions can potentially be used for performing oxygen assays in accordance with the subject method. The copolymerization of two vinyl monomers, specifically acrylamide and bisacrylamide, is discussed in detail by way of example only. Other compounds that can be used for oxygen assays will be mentioned below and still others will be obvious to skilled chemists.

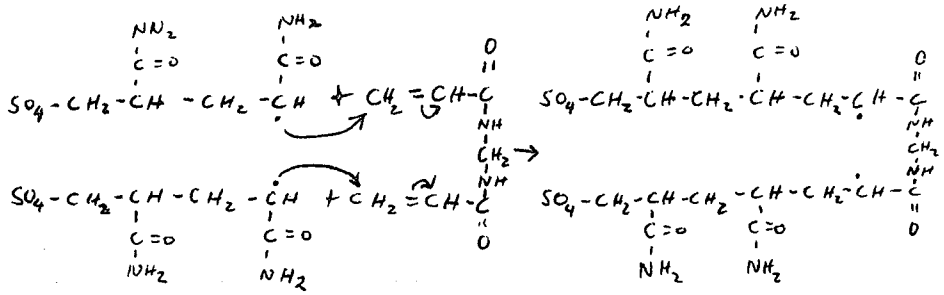
Polymerization of acrylamide is initiated by sulfate radical ions (SO_4^-.) generated by the scission of a weak **0—0** linkage in persulfate ions ($S_2O_8^=$) with the activation energy advantage provided by a reduction activator, bisulfite (HSO_3^-). The sulfate radical ion attacks the double bond of an acrylamide molecule as follows to generate an acrylamide radical.



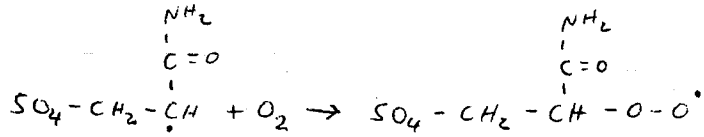
In the absence of oxygen, polymerization would then occur through propagation steps based on acrylamide radicals attacking fresh acrylamide molecules to yield new radicals which would then attack further fresh acrylamide molecules, and so forth in a chain reaction, with the polymer solution becoming progressively more viscous and ultimately gelling as polymerization proceeds.



The firmness of the polymer thus formed is increased by adding a certain proportion of N,N'-methylenebisacrylamide, which permits cross-linking of polymer chains:



In the presence of oxygen, the acrylamide radical reacts with molecular oxygen in preference to another molecule of acrylamide to yield a peroxy radical.



This, in contradistinction to the acrylamide radical, is relatively unreactive. Thus the acrylamide, rather than lengthening its polymer chain, is tied up as long as oxygen is present. The oxygen is used up, of course, as it is incorporated into acrylamide molecules. Once its concentration in the medium becomes sufficiently small, the peroxy radicals and acrylamide radicals will again react with acrylamide, and the more reactive acrylamide-ended radicals will react quickly in chain-lengthening steps. (The peroxy radicals are, of course, slow to react. However, once a peroxy radical does react with an acrylamide molecule, it forms an acrylamide-ended radical and thus continues to react rapidly.) The net result of all this is a lag period during the course of the polymerization reaction, during which oxygen is used up, followed by rapid polymerization. The transition between these phases can be quite abrupt. The more oxygen present in relation to acrylamide, the longer the lag period.

Three solutions are generally used in the acrylamide oxygen assay in addition to the blood sample: a monomer solution, a persulfate solution and a bisulfite solution.

1. Monomer Solution: This is prepared according to the formula:

Acrylamide **6.12**g
Bisacrylamide **0.308** g
Distilled Water **20** ml

and degassed 8 min in 30 ml syringes with a rotary vacuum pump. If the liquid to be assayed varies in pH, it is beneficial to buffer the monomer solution by dissolving 0.477 g of HEPES (N-2-hydroxyethylpiperacine-N-2-Ethanesulfonic acid (availabe from Calbiochem, Los Angeles), or other buffer, in a portion of the distilled water along with the acrylamide and bisacrylamide, titrating to pH 7.4, adding the remainder of the distilled water and degassing

as described. The degassing step can be omitted if a new standard curve is prepared daily as described below. The acrylamide appears quite stable on storage. It should be kept stoppered, refrigerated and in the dark to slow any spontaneous polymerization. Before use the solution is rewarmed to room temperature (23°).

2. Bisulfite Solution: For banked heparinized (anticoagulated) blood, 0.123 g sodium bisulfite is dissolved in 50 ml of degassed distilled water and transferred to evacuated glass tubes. For fresh heparinized blood, the amount of bisulfite is increased to 0.160 g. Degassing of the water can be omitted as described below. The solution is prepared fresh daily. These quantities were empirically determined to be optimum.

3. Persulfate Solution: 0.154 g of ammonium persulfate is dissolved in 50 ml distilled water and the solution is degassed. The degassing can be omitted if a new standard curve is prepared daily. The solution is prepared fresh daily.

Referring now to FIGS. **1** and **2**, the acrylamide assay is run manually in a 1 ml plastic tuberculin syringe mixing chamber **21**, calibrated in 0.01 ml divisions, containing a glass bead **22** that is 4 mm in diameter and serves to mix reactants and to detect the formation of a polymer gel by monitoring viscosity The plunger **23** is moved to the 0.40 ml mark and monomer solution is injected to the tip of the syringe orifice **24**. The solution is injected from a hypodermic needle **25** on another syringe **26** as shown in FIG. **1**. Note that once fluid is in the syringe **21** and a liquid-gas interface **27** appears, new fluids are inserted below that interface. The plunger **23** is then moved to the 0.60 ml mark and persulfate solution is injected, again to the tip of the syringe orifice **24**. The plunger **23** is moved to the 0.70 ml mark and 0.10 ml blood sample with heparin is added, to the tip of the orifice. The present contents of the syringe comprise the test liquid. The syringe **21** is capped with a cap **28** and mixed by inversion 10–15 times. Hemoglobin is liberated from red cells by this procedure. The cap **28** is removed, the plunger lowered to below the 1.0 ml mark, and 0.30 ml of the bisulfite activator solution, premeasured in a 1 ml syringe, is rapidly injected to begin the reaction and production of the product. Timing with a stopwatch begins as this injection is concluded. The air bubble at the top is expressed, the syringe **21** capped and held as shown in FIG. **2** (the syringe **21** is held as shown in FIG. **2** to avoid warming the solution by body heat) and mixed by inversion while observing the glass bead **22**. Timing stops when the glass bead **22** becomes immobilized (as shown in the right syringe) by an increase in viscosity showing the product has completed the formation of a polymer gel. This is preceded by a 10 second warning period of increasing viscosity.

A blank determination of a base reference time is performed identically with 0.10 ml normal saline (physiological saline solution) in place of blood. The polymerization time observed for the blank is subtracted from each experimental polymerization time.

The polymerization reaction is exothermic. With a thermal probe in the reaction syringe, the temperature is observed to rise 4°–5°within the 15 seconds preceding gelation; after gelation the temperature rises more rapidly to about 20° over the starting level. Thus, temperature could be used as in indication of the gelation.

Oxygenated blood, initially bright red in the reaction syringe **21**, slowly darkens to a deep violet as the polymerization reaction proceeds. The bright red color can be restored to the gel subsequently by exposure to oxygen, suggesting that the initial color change is due to deoxygenation of the oxyhemoglobin. Thus, color is indicative of gelation.

Referring next to FIG. **3** there is typical standard of comparison that is a graph **31** that is a curve **32** with an upper end point **33** and a lower end point **34**. Comparison of results of oximetry by the subject method and by conventional techniques has shown a plot of the logarithm of time v. oxygen content is linear to 22 ml O_2/100ml blood.

There was some day-to-day variation in the polymerization time — oxygen content curves obtained with non-degassed acrylamide making it desirable to find a rapid means to establish a daily standard curve. This was most readily accomplished by computing the oxygen content of a fully oxygenated blood sample from the hemoglobin content (1.34 ml O_2/ gm Hb) plus dissolved oxygen, and using this sample to establish the upper end **33** of the curve **32**, while using a saline blank to establish the lower end **34** of the curve. The lower end **34** of the curve **32** does not go through the origin because saline is not a true blank. Only blood with no oxygen would be. This is no problem, however, inasmuch as the curve **32** is linear. Corrected polymerization time is sample polymerization time minus blank polymerization time.

Use of manual mixing and timing and hand-held syringes **21** for the oxygen assay, though it is simple and inexpensive, opens the method to a substantial amount of deviation and error.

It was felt imperative to develop a semiautomatic system that would eliminate subjective estimation of the end point of the reaction fna minimize the variability of mixing vigor and temperature, so more reproducible and accurate results could be obtained.

Accordingly, the mixing apparatus with an electromagnetic monitor system, as shown in FIG. **4**, was devised. The reaction mixing chamber **41** consists of a 10 ml plastic syringe **42** containing a ½ inch plastic-coated magnetic stirring bar **43**. The nozzle end of the syringe barrel **42** is wrapped with a 12 turn coil **44** of insulated electrical hookup wire within the magnetic field of the bar **43**. The ends of this coil **44** are connected to an amplifier **45**. The syringe nozzle access opening is plugged with a rubber injection port **46** after being filled with 1.2 ml of acrylamide, so that no air space remains and the solution is isolated from the atmosphere. Subsequently, all reagents are injected through this rubber port **46** so that no air is admitted to the chamber **41** at any time. The syringe **42** is clamped horizontally over a magnetic stirrer motor **47** which is switched on and adjusted to low speed (about 75 rpm). The amplifier **45** is connected to a monitor **48** that can be a chart recorder. It should be emphasized that the monitor can also be a digital clock responsive to the amplifier **45**.

As the magnetic stirrer **47** causes the stirring bar **43** to turn, an electromotive force is generated in the coil **44** and recorded by the chart recorder. Measurement indicates this is about 1 mV peak amplitude. Persulfate, blood and bisulfite are injected through the injection port **46** in the usual sequence, with all volumes 3 times that for the manual method. When a gel forms, the magnetic stirring bar **43** is immobilized by the increased viscosity and the alternating voltage drops markedly to a low level produced by the magnetic field

of the stirrer **47.** The voltage drop occurs over about 2 seconds. The time interval from the addition of bisulfite can easily be read off the chart paper if the paper speed is known.

To secure accurate temperature control, a jacketed water bath assembly **51** has been designed and is shown in FIG. **5.** This assembly permits 10 ml syringes to be placed rapidly in position with respect to a permanently installed coil **52** in a lucite tube **53** over the stirrer motor **47.** There are also jackets **54** to store persulfate, bisulfite and blood and assure they are at the appropriated temperature. A divider **55** separates the bath **51** into a storage chamber **56** and a reaction chamber **57.** Separate inlets **58** and outlets **59** are provided for each chamber **56, 57.** Separate chambers are supplied because the reaction is exothermic and thus the reaction chamber **57** requires a greater water flow to stabilize. A thermometer **61** indicates the temperature in the reaction chamber **57.**

It should be noted that the syringe apparatus shown in FIG. **4** is useful in carrying on any reaction requiring atmospheric isolation and/or viscosity monitoring and the bath **51** shown in FIG. **5** is useful in those reactions if the temperature must be controlled.

Other reagent systems are suggested below. The final choice of any one system must be made by the user of the method in light of his preferences and the liquid to be assayed.

Bisulfite need not be used. Other reduction activators of persulfate include ferrous ions and ferrocyanide.

Blood, too, is a weak reduction activator for persulfate. Consequently, if time is not of the essence, a test solution of blood and a monomer can be used and the addition of the activator persulfate solution initiates a slow polymerization process that is completed in several hours. The natural consequence of the above is that if a reduction activator is used it must be added quickly after the addition of the blood, and the time delay between addition of the blood and the reduction activator should be uniform from test to test.

Reduction activators can be eliminated. A test solution of blood, or another liquid to be assayed, and a monomer can be tested with an activator liquid comprising a compound such as benzoyl peroxide that spontaneously generates free radicals and thus starts polymerization.

Furthermore, other uses of the method are evident. A few other uses are suggested below.

The oximetry of fluorocarbons has been a difficult process. However, it has been found that fluorocarbons can be assayed quickly by the subject method, and a plot of log time vs. oxygen content is linear up to 22 ml. oxygen/100 ml. fluorocarbon. In addition, the high oxygen solubility of fluorocarbons permits other uses such as the one following.

Measurement of tracheal and atmospheric oxygen should be possible if the gas is first equilibrated with blood or fluorocarbon and the fluid then analyzed by the acrylamide method. Solubility data for oxygen in the liquid in question would permit extrapolation back to oxygen tension in the gas phase, most easily if the solution obeys Henry's law of gas solubility as do fluorocarbon emulsions.

With suitable adjustments of activator and monomer concentrations (to be determined empirically) oxygen content of water samples could be determined rapidly and in the field if necessary.

With a suitable microassay based on the acrylamide principle, for oxygen in aqueous solution, any enzymatic reaction which consumes or produces oxygen could be assayed if the reaction chamber is sealed and sampling can be performed anaerobically. Aliquots of the enzyme-substrate mixture could be removed at intervals and assayed for oxygen content to provide an index of the rate of reaction.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is to be understood, therefore, that the invention can be practiced otherwise than as specifically described.

What is claimed is:

1. A method of measuring the oxygen content of liwuids comprising the steps of:

mixing a test liquid containing oxygen with an activator liquid to produce a polymer by a free radical initiated polymerization reaction that is inhibited by oxygen in said test liquid;

isolating said test liquid and said activator liquid from any gas phase during said mixing step; and

timing said polymerization reaction to determine the amount of oxygen initially present in said test liquid and consumed during said reaction.

2. A method according to claim **1** including a degassing step for removing gases from said activator liquid before said mixing step.

3. A method according to claim **1** wherein the temperature of said liquids is regulated during said mixing and timing steps.

4. A method according to claim **1** where said test liquid is water.

5. A method according to claim wherein said test liquid comprises a fluorocarbon.

6. A method according to claim **5** comprising an equilibrating step to equilibrate said fluorocarbon with a gaseous atmosphere before said mixing step.

7. A method according to claim **1** wherein said timing step comprises a viscosity monitoring step.

8. A method according to claim **7** comprising the step of comparing the measure of time yielded by said timing step to a standard of comparison for determing the amount of oxygen in said test liquid.

9. A method according to claim **8** wherein said polymerization comprises gelation and said polymer is a gel.

10. A method according to claim **8** wherein said standard of comparison is a graph comprising a curve with upper and lower end points.

11. A method according to claim **10** wherein said upper end point is located by oximetery of a fully oxygenated blood sample.

12. A method according to claim **10** wherein said lower end point is located by oximetery of a physiological saline solution.

13. A method according to claim **8** wherein said test liquid comprises a monomer solution and said activator liquid comprises free radical ions.

14. A method according to claim **13** wherein said test liquid further comprises blood.

15. A method according to claim **14** wherein said test liquid further comprises an anticoagulant.

16. A method according to claim **15** wherein said test liquid further comprises a buffer.

17. A method according to claim **8** wherein said mixing step comprises mixing a reduction activator with

said liquids for producing sulfate radical ions in said activator liquid.

18. A method according to claim **17** wherein said reduction activator comprises bisulfite and said monomer comprises a vinyl monomer and said activator liquid comprises persulfate ions.

19. A method according to claim **18** wherein said reduction activator comprises sodium bisulfite and said vinyl monomer is acrylamide and said activator liquid comprises ammonium persulfate.

20. A method according to claim **18** wherein said polymerization includea copolymerization.

21. A method according to claim **20** wherein said test liquid further comprises bisacrylamide.

22. A method of measuring the oxygen content of liquids comprising the steps of:

providing an oxygen containing test liquid and an activator liquid;

degassifying said activator liquid;

mixing said test liquid with said activator liquid to produce a polymer by a free radical initiated polymerization reaction that is inhibited by oxygen in said test liquid; and

timing said polymerization reaction to determine the amount of oxygen initially present in said test liquid and consumed during said reaction.

23. A method of measuring the oxygen content of fluorocarbons comprising the steps of:

mixing a fluorocarbon test liquid containing oxygen with an activator liquid to produce a polymer by a free radical initiated polymerization reaction that is inhibited by oxygen in said test liquid; and

timing said polymerization reaction to determine the amount of oxygen initially present in said fluorocarbon test liquid and consumed during said reaction.

24. A method of measuring the oxygen content of water comprising the steps of:

mixing a test sample of water with an activator liquid to produce a polymer by a free radical initiated polymerization reaction that is inhibited by oxygen in said test sample; and

timing said polymerization reaction to determine the amount of oxygen initially present in said test sample and consumed during said reaction.

25. A method according to claim **24** comprising the step of isolating said liquids from any gas phase during said mixing step.

* * * * *