Acrylamide Polymerization: New Method for Determining the Oxygen Content in Blood

Abstract. *A new principle for determining the oxygen content of 0.1-milliliter blood samples has been developed, based on measurement of the delay in gelation during copolymerization of acrylamide and bisacrylamide initiated by free radicals. The logarithm of this time interval is linearly proportional to the oxygen content of the blood sample over the range 0 to 22 milliliters of oxygen per 100 milliliters of whole blood. Physiological variations of* p*H and* pCO_2 *do not affect the sensitivity of the assay.*

Oxygen is known to inhibit the polymerization of acrylamide initiated by free radicals (*1*). We observed this phenomenon while preparing acrylamide gels for use in purifying tumor angiogenesis factor (*2*), and later used this property to measure the oxygen content of small blood samples. To our knowledge such a principle of oxygen analysis has not been described previously.

Three reagents are required. The monomer solution is a combination of 6.12 g of acrylamide and 0.308 g of *N,N'*-methylenebisacrylamide dissolved in 20 ml of 100 m*M* *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid, which is titrated to *p*H 7.40 with 1*N* NaOH, and then degassed with a rotary vacuum pump for 8 minutes. The resulting solution was stored in plastic syringes fitted with a 22-gauge hypodermic needle and capped with a rubber stopper. The other two solutions must be prepared fresh daily: (i) $3.07 \times 10^{-2}M$ sodium bisulfite ($NaHSO_3$), prepared in degassed water and stored in evacuated test tubes, and (ii) $1.35 \times 10^{-2}M$ ammonium persulfate [$(NH_4)_2S_2O_8$], degassed and stored in syringes as described above for acrylamide.

Blood samples were drawn from the venous and arterial catheters of 11 postoperative cardiac patients, and from three normal subjects by venipuncture; samples were immediately placed in heparinized syringes on ice. Several samples were subdivided into aliquots with a wide range of oxygen contents by the mixing method of Lenfant *et al.* (*3*). Before analysis each sample was warmed to 23°C in a water bath.

The oxygen content of each sample was determined within 2 hours after being drawn by the manometric method of Van Slyke and Neill (*4*), and the acrylamide assay was performed in duplicate by methods described below.

1) Glass bead method for determining gelation end point. The reaction vessel is a 1-ml plastic syringe containing a 4-mm glass bead that serves to mix the reactants and to detect gelation. The plunger is moved to the 0.40-ml mark, and acrylamide solution is injected to the tip of the syringe outlet (Fig. 1A). The plunger is then moved to the 0.60-ml mark, and persulfate solution is injected, again to the tip of the syringe outlet. The plunger is moved next to the 0.70-ml mark, and 0.10 ml of whole blood is added to the tip of the syringe outlet. The syringe is capped, and the contents are mixed by 15 inversions. Erythrocytes are hemolyzed by this maneuver. The syringe is uncapped, the plunger is moved to about the 1.0-ml mark, and 0.30-ml of bisulfite solution, measured first in a 1-ml syringe, is rapidly injected. As this injection is concluded, timing with a stopwatch begins. The residual air is quickly expressed, the syringe is capped, and the mixing is continued by slow inversion while observing the glass bead. Timing stops when the bead becomes immobilized by the formation of a gel (Fig. 1B) (*5*). This end point is a precise one, and takes place after a 5- to 10-second warning period of increasing viscosity. Such a determination is performed in an identical way on a blank, except that the blood sample is omitted; the polymerization time for the blank is subtracted from the time determination on the sample containing blood. The true blank for this system is a blood sample with an oxygen content of zero; in practice this is difficult to obtain, and so we chose to simplify the method, recognizing that the time determination for our blank would be different from that of the true blank. The reaction mixture containing blood changes in color from bright red to violet as the polymerization proceeds. The red color can be restored by exposing the gel to O_2, a suggestion that the initial color change is due to deoxygenation of oxyhemoglobin.

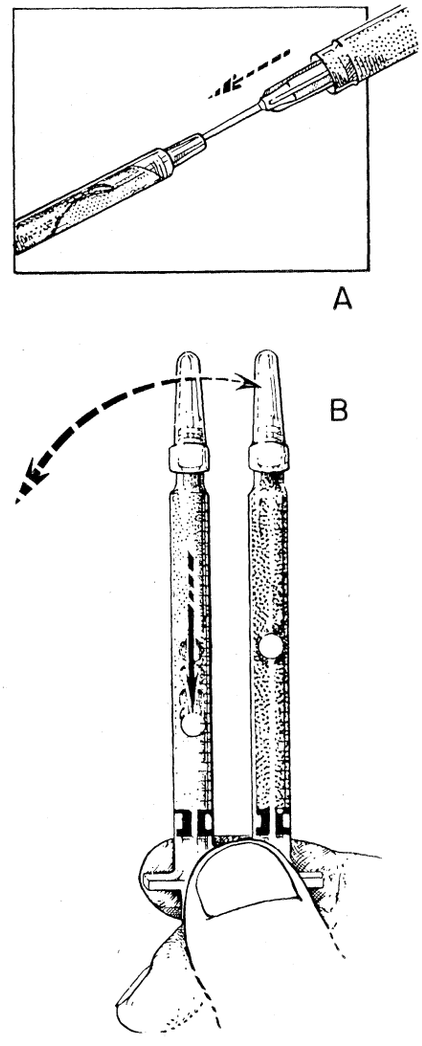
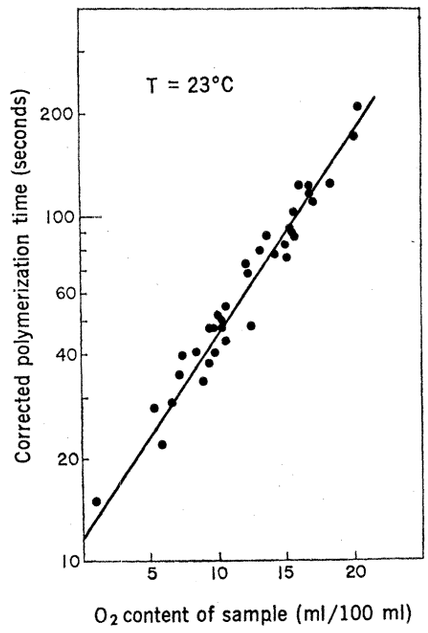


Fig. 1 (A). Method of adding reactants to the 1-ml plastic syringe. (B) Polymerization reaction in progress. The end point of timing has been reached in the syringe at right. Double-headed arrow indicates direction of shaking.

The logarithm of polymerization time thus obtained was found to be linearly proportional to oxygen content over the range of 0 to 22 ml of O_2 per 100 ml of whole blood (Fig. 2). The duplicate polymerization times on which each data point is based agreed usually within 2 seconds. The standard deviation of individual data points from the mean line was 0.92 ml of O_2 per 100 ml of whole blood. This substantial variability is attributable to thermal and mixing variations, and to variable exposure of the reaction mixture to atmospheric oxygen. The large standard deviation was reduced by 50 percent when these factors were better controlled with a semiautomatic electronic method of detecting gelation, and with a constant-temperature bath.

2) Semiautomatic method for determining gelation end point. A method recently devised by Harmel and Folkman (*6*) utilizes the same principle but takes advantage of the fact that a magnetic stirrer spinning in the acrylamide solution will induce an alternating voltage in a surrounding wire coil. When

Fig. 2. Acrylamide polymerization time determined by the glass bead method, and corrected for a blank, plotted semilogarithmically as a function of oxygen content of blood samples.



the polymer solution gels, the stirrer is immobilized, and the alternating voltage is suddenly reduced. An electronic timer is triggered automatically by this voltage drop and gives a direct readout of gelation time (*7*). This method has been used to measure polymerization times for 40 blood samples with varying *p*H, hematocrit, and oxygen content. The standard deviation is reduced to 0.42 ml of O_2 per 100 ml of whole blood (Fig. 3). This semiautomatic method is rapid and dependable; the oxygen content of 20 samples can be determined in 1 hour.

The lines obtained from the results of both methods do not pass through the origin because the blank subtracted from each point is not a true blank. When the blood is omitted, the concentration of acrylamide is increased about 10 percent; this produces a shorter polymerization time. When saline was used as a blank the polymerization time was 2 to 5 seconds longer than when the blood was omitted altogether, but was still shorter than that of blood with zero oxygen content by 12 to 13 seconds. Whether or not the plot passes through the origin does not matter, as long as the plot is linear.

In separate experiments we noted that variation in the *p*H of the blood sample from 7.0 to 8.0, and variation in the pCO_2 from 0 to 100 mm-Hg do not affect the polymerization times obtained with reagents prepared as described. The reaction is sensitive to temperature, however, with polymerization occurring more rapidly at higher temperatures. Precautions should be taken, therefore, so that standard and experimental samples are tested at a specific temperature, and that the inadvertent transfer of body heat to the reaction syringe is avoided. Satisfactory results can be obtained if degassing of reagents in vacuum is omitted, although this introduces substantial day-to-day variation in the absolute magnitude of polymerization times observed, requiring daily preparation of a standard curve. This can be done most conveniently by measuring the polymerization time of a blood sample fully saturated with oxygen (by equilibration with air), the O_2 content of which is calculated easily on the basis of hemoglobin concentration (1.34 ml of O_2 per gram of Hb) with a correction added for dissolved oxygen.

With the glass bead method, as well as with the semiautomatic method, the time required for injection of the bisulfite solution is very important, and can be accomplished comfortably in about 30 seconds, before mixing begins. The highest accuracy is obtained when this interval is kept constant at approximately 30 seconds.

The greatest precision was obtained when all of the samples had the same hematocrit; the standard deviation of individual data points from the graph of a line of the means was 0.36 ml of O_2 per 100 ml of whole blood. When the hematocrit of the samples differed over a range of 10 percent, the standard deviation was slightly higher (0.42 ml of O_2 per 100 ml of whole blood). However, when the hematocrit of the samples differed by more than 10 percent, separate regression lines could be plotted. For a given oxygen content, the lower the hematocrit the more prolonged the polymerization time. This was not due to incomplete hemolysis at the higher hematocrits; prior hemolysis of all samples with Triton-X had no effect. However, when red cells were suspended in saline at varying hematocrits, there was only one regression line as the oxygen content increased. This implied that the "hematocrit effect" on polymerization was due to something in the plasma rather than in the red cells. Therefore, if this method is used for multiple samples with the same hematocrit, for example, during cardiac catheterization or during the construction of an oxyhemoglobin dissociation curve, all of the points fall on a single line. If, however, the hematocrits vary over a wide range, the greatest accuracy is obtained when one line is drawn for each hematocrit, even though these lines are very close together and converge at the ordinate.

We anticipate the application of the acrylamide principle to rapid generation of oxyhemoglobin dissociation curves, to measurement of oxygen content at the bedside, to determination of differences in oxygen content of arteriovenous blood during cardiac catheterization, to determination of oxygen content of other solutions such as fluorocarbons or water, to measurement of tracheal or atmospheric oxygen (with an intermediary fluid equilibration), and to assay of enzymatic reactions which consume or produce oxygen.

Although the glass bead method is rapid and useful at the bedside, the semiautomatic method is more precise and, therefore, has wider applicability. It might also be possible to eliminate the stopwatch and the electronic timer by causing liquid acrylamide to flow through a calibrated capillary tube and stop at the moment of gelation.

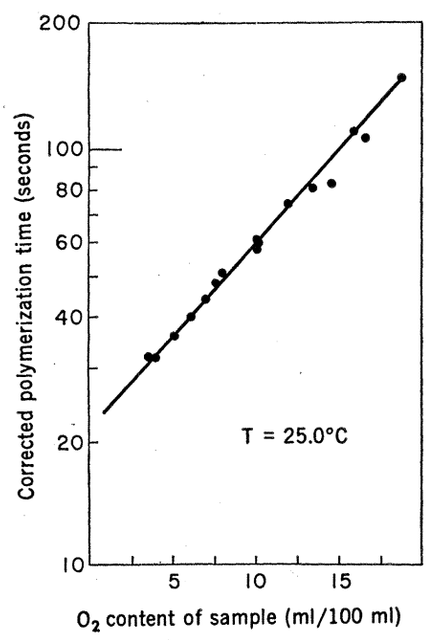


Fig. 3. Acrylamide polymerization time determined by the semiautomatic method and corrected for a blank, plotted semilogarithmically as a function of oxygen content of 16 blood samples with hematocrits varying from 30 to 40 percent.

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References and Notes

1. S. Raymond and L. Weintraub, *Science* **130**, 711 (1959).
2. J. Folkman, E. Merler, C. Abernathy, G. Williams, *J. Exp. Med.* **133**, 275 (1971).
3. C. Lenfant, P. Ways, C. Aucutt, J. Cruz, *Resp. Physiol.* **7**, 7 (1969).
4. D. Van Slyke and J. Neill, *J. Biol. Chem.* **61**, 523 (1924).
5. We refer to this time interval as "polymerization time." We recognize that the end point we observe is gelation, which occurs at one particular degree of polymerization.
6. R. Harmel and J. Folkman, in preparation.
7. The semiautomated device for electronic readout of gelation was built for us by R. Jeffery and F. Fillippone at the C. S. Draper Laboratory of the Massachusetts Institute of Technology, Boston.

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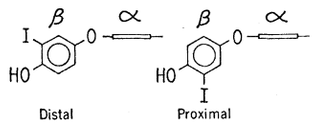
Thyroxine-Binding Globulin: Specificity for the Hormonally Active Conformation of Triiodothyronine

Abstract. *The conformational requirements for binding of triiodothyronine to thyroxine-binding globulin were investigated with triiodothyronine analogs having restricted rotation at the ether bond. Although it has been reported that the predominant conformation of triiodothyronine carries the 3′ iodine in a position proximal to the phenylalanine ring, the analog for the distal, hormonally active orientation of the 3′ iodine is more effective in displacing triiodothyronine and thyroxine from thyroxine-binding globulin. The lower binding affinity of thyroxine-binding globulin for triiodothyronine as compared to thyroxine may be explained by specificity of the binding site for the less abundant conformation of triiodothyronine.*

The single β-ring iodine of triiodothyronine (T_3) imparts a degree of asymmetry and conformational variability that is not shared by thyroxine (T_4). Both hormones must position their phenyl rings in mutually perpendicular planes to minimize steric interaction. For T_3 but not T_4, this allows two distinct conformations, one with the 3′ iodine distal to the α ring, the other with the 3′ iodine proximal to the ring. The difference between



these two conformations is of biological significance because it appears that hormonal activity is restricted to the distal orientation of the 3′ iodine (*1*). Although it seemed likely that this would prove to be the predominant conformation of T_3, x-ray crystallographic studies revealed a proximal orientation for the 3′ iodine, and this conformation was also calculated to have a lower molecular energy (*2*). Rotation at the ether bond is permitted; therefore, in free solution both conformations probably exist in an equilibrium favoring the proximal 3′ iodine orientation. However, less than 1 percent of the total T_3 in human serum is in free solution. The remainder is nonconvalently bound to thyroxine-binding globulin (TBG) and albumin.

Since the binding site on TBG shows stereochemical specificity (*3*), it was of interest to determine whether the site distinguishes the distal and proximal orientations of the 3′ iodine. Such binding specificity would strongly affect the equilibrium between the two conformations.

The conformational requirement for TBG binding was investigated with DL-3,5,-diiodo-2′,3′-dimethylthyronine as the analog for the distal 3′ iodine position and DL-3,5,-diiodo-2′5′-dimethylthyronine as the analog for the proximal 3′ iodine position. These analogs were synthesized by Jorgensen and his colleagues (*4*) to determine the relative hormonal activity of 3′ iodine in the distal and proximal positions. In these compounds rotation of the phenyl rings at the ether bond is blocked by the 2′ methyl group. Because of difficulties in synthesis, a methyl group was also introduced as the steric equivalent for the distal or proximal positions of iodine in the β ring. The distal analog is hormonally active, having 50 percent as much goiter-suppressing activity as l-T_4 and 13 percent as much calorigenic effect as l-T_3 (*5*). The proximal analog shows only 1 to 2 percent as much activity as the distal analog in these assays.

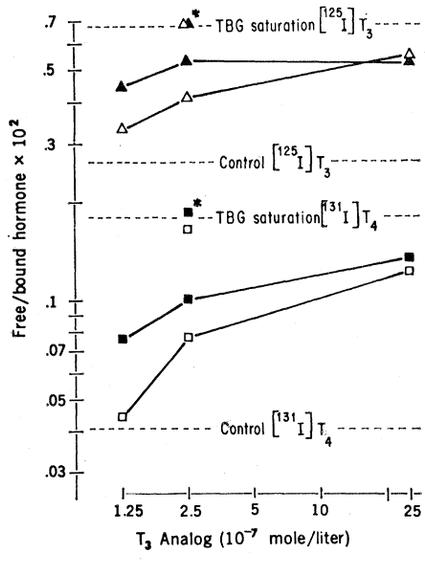


Fig. 1. Effect of analogs for the distal and proximal orientations of the 3′ iodine on ratios of free to bound T_3 and T_4 in dilute (1 : 100) human serum. For binding of [^{125}I]T_3, symbols are ▲, data for the distal analog, and △, data for the proximal analog; for binding of [^{131}I]T_4, symbols are ■, data for the distal analog, and □, data for the proximal analog. The TBG saturation lines indicate the ratios obtained when $1.28 \times 10^{-6}M$ unlabeled T_3 (enough to saturate TBG) was present in addition to the labeled hormone indicated. The pairs of data points marked by * were obtained when this concentration of unlabeled T_3 was present in addition to analog and labeled hormone. The concentrations of radioactive T_3 and T_4 were approximately $4 \times 10^{-10}M$.

The low total binding capacity of TBG (about $3 \times 10^{-7}M$ T_4 in normal human serum) made it difficult to directly measure analog bound to TBG. Therefore, the binding affinities of the oriented T_3 analogs were compared indirectly by their displacement of isotopically labeled T_3 and T_4. The T_3 analogs were dissolved in alkaline ethanol. Ratios of free hormone to bound hormone were determined by ultrafiltration of a 1:100 dilution of pooled human serum in 0.15M phosphate buffer at pH 7.4 and 37°C. This high dilution of serum increases the sensitivity of the system to competitive binding and was used to minimize the quantity of T_3 analogs required. Aliquots (25 μl) of appropriate dilutions of the stock solutions of T_3 analogs were added to 21 ml of dilute serum in phosphate buffer. An equal quantity of diluent was added to control serum. Isotopically labeled T_4 and T_3 were each separated from contaminants by a preliminary dialysis and then added to the dilute serum in a concentration no greater than 0.03 μg per 100 ml. The ultrafiltration procedure and subsequent analysis have been described (*6*). Protein electrophoresis