

Analytical Techniques for Cell Fractions

X. High-Pressure Ninhydrin Reaction System

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Automated bioanalytical systems are usually scaled on the basis of available components since the initial objective is, generally, to prove underlying principles rather than to construct a system having optimum performance. As additional devices are developed, the problems of resolution, accuracy, sensitivity, size, and maintenance become of increasing interest. For both research purposes and clinical use, small reliable instruments are required. We are interested in exploring the factors that appear to be limiting in the development of one class of such instruments, namely, those depending on ion exchange. While attention in this laboratory has previously been centered on the development of automated systems for nucleotides and related compounds (1-3) and for sugars (4), our interest is now directed toward the problem of reducing the size and analysis time for the most widely used system: the amino acid analyzer of Spackman, Stein, and Moore (5), Piez and Morris (6), and Hamilton (7).

Theoretical (8) and experimental (7) studies on ion-exchange separation have indicated that a limiting factor is diffusion of the solutes to be separated into and out of the ion-exchange bead. Interest has therefore focussed on means for shortening the distance through which solutes have to diffuse, either by decreasing the volume of the ion-exchange bead (7) or by confining the exchange resin to the surface of an impermeable bead (9). The limiting resolution will probably be obtained by using both approaches simultaneously, which will result in high back pressures. Unfortunately, high-pressure pumps having accurate deliveries at very low flow rates are generally large and difficult to build and maintain,

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which defeats one of the basic objectives of miniaturization. With miniaturized systems, considerable thought must be given to the problems of handling small volumes at low flow rates, and of maintaining resolution throughout the system. The ninhydrin reaction as currently done involves passing the reaction mixture through a long Teflon coil. The decrement in resolution that occurs can be serious for peaks that are eluted in a very small volume. It appeared to us that the problem of accurately metering small volumes at high pressure and of handling them throughout the system, and the problem of increasing the rate of ninhydrin reaction in order to reduce dwell time (and therefore volume in the reaction coil to thus not lose resolution at this point), were related. Both problems could be solved simultaneously by (a) pressurizing the entire system all the way past the flow cell used for absorbance measurement, (b) controlling flow through the whole system from the low-pressure end by withdrawing fluid from that end at a constant rate, the injection of reagents being automatically coordinated with this rate, and (c) controlling the pressure at the high-pressure end by feedback of pressure information from the low-pressure end. In this paper we are chiefly concerned with the question: *Can the ninhydrin reaction rate be appreciably increased by increasing the temperature and the pressure in a closed flowing system?* However, since the results bear directly on the problem of designing miniature systems, the over-all design requirements and concepts are discussed first, and experimental work on a pressurized ninhydrin system is discussed second.

PROBLEMS IN MINIATURIZATION OF THE AMINO ACID ANALYZER

A diagrammatic representation of the system under development is shown in Figure 1. Gas pressure from an inert gas cylinder 1 is fed to a liquid piston (hydraulic accumulator) 2, which transfers this pressure through small tubing to a buffer chamber or chambers 3. There may be several of these, with appropriate valving to allow buffer changes as the run progresses. Sample is introduced at valve 5 and flows (followed by buffer) through the ion-exchange column 6. The column effluent is mixed with ninhydrin reagent solution from syringe reservoir 7, and the mixture flows through a short heated coil 8 for color development and then through a pressurized colorimeter 9, which provides a signal to the strip-chart recorder 13. The effluent from the colorimeter flows past a pressure sensor 10 (which acts on pressure-regulating valve 4 to adjust the pressure on the column to provide a selected pressure level at 10) and then into the collecting syringe 11. The collecting syringe is disposed in such a manner that its piston moves at the same lineal rate (controlled by a small drive motor 12) as does the piston of the ninhydrin syringe

but in an opposite direction for flow. The amount of reagent solution being injected thus bears a constant ratio to the amount of liquid coming from the column. The rate of flow through the column and through the analytical system is constant, but this may matter much less than having the internal contributions from the reagent-injecting system and the column in constant ratio to each other as described. The actual ratio is determined by the relative cross-sectional areas of the pistons. It is

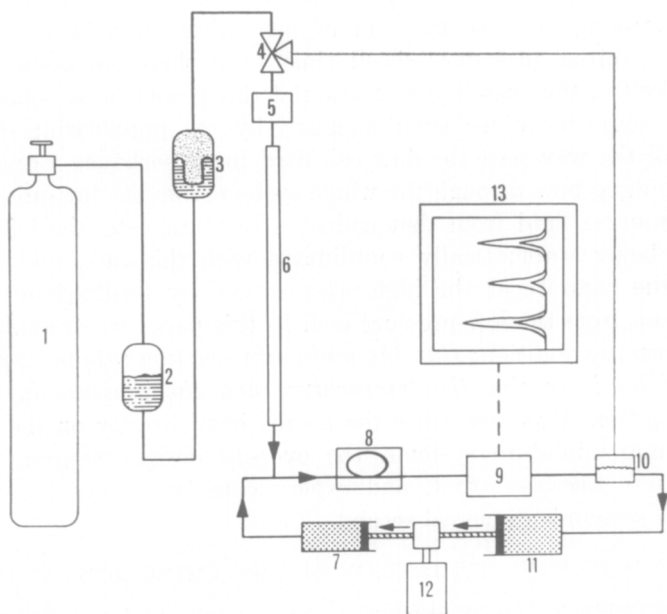


FIG. 1. Schematic diagram of miniaturized amino acid analyzer. Parts are indicated as follows: (1) source of gas pressure; (2) hydraulic accumulator; (3) buffer chamber or chambers; (4) pressure-control valve; (5) sample introduction valve; (6) ion-exchange column; (7) ninhydrin syringe; (8) heated color-reaction coil; (9) colorimeter; (10) pressure sensor which controls pressure through 4; (11) effluent-collecting syringe; (12) syringe-drive system; (13) strip-chart recorder.

seen that flow is controlled separately from pressure in the sense that, even with excess pressure at the head of the column, flow rate through it would be constant (since total flow is restricted by the motion of the receiving syringe). This would result, however, in unnecessary pressure in the low-pressure end of the system, and so pressure to the column is controlled to maintain just the proper flow in the column and a chosen constant pressure at the low-pressure end.

Various safety devices such as liquid interlocks and flow restrictors to

avoid spurling of liquids in case of failure, as well as valving for refilling the buffer chambers and the valving and mechanical arrangement for quick refilling of the ninhydrin syringe and emptying of the reservoir syringe, are not shown here.

As discussed above, one of the chief points at which it was feared resolution could be lost was in the ninhydrin reaction line. Efforts were therefore focussed on methods for reducing the volume of the reaction system and the color development time to a minimum, and to these ends the effects of time and temperature on the ninhydrin reaction in a closed system were studied.

EFFECTS OF TIME AND TEMPERATURE ON THE NINHYDRIN REACTION

Glass-Teflon Reaction System

The effect of temperatures above 100° can be studied only at increased pressure. A glass reaction vessel was therefore constructed and filled with a high-boiling-point liquid (Freon E-3, b.p. 150°, Dupont)³ so that temperatures up to 150° could be obtained. Coils of Teflon tubing (AWG 22) of different lengths were arranged in the bath. Two syringes, driven by lead screws, were filled—one with a ninhydrin reagent (see *Reagents* section below) and the other with a citrate buffer (*Reagents* section) containing a low concentration of an amino acid mixture. The two solutions flowed to a mixing tee and then into the Teflon reaction coil. The effluent from the coil flowed to a pressurizable flow cell in a Beckman DB spectrophotometer, and then out to a pressure-adjustable waste vessel.

The satisfactory quality of the ninhydrin reagent was checked by mixing the buffer and reagent and heating them together in a test tube in boiling water. However, when the solutions were run through the reaction bath at 100° no color was observed. When the Freon was removed and replaced with water, the expected blue color appeared. This effect seemed to be due to light fractions of the Freon penetrating the coil and inhibiting the ninhydrin reaction, as was suggested by the fact that a temporary inhibition of color development occurred when small volumes of Freon E-3 were added to the water-filled glass reaction vessel. The color returned after some vapor coming from the Freon had been released through the reflux condenser.

³ Mention of commercial products in this paper is meant to be informative rather than restrictive. Other products of similar characteristics may work equally well.

Metal and Teflon System

Because of the difficulties with the system mentioned above, a system employing a stainless-steel reaction coil on a heating block, with Teflon tubing leading to and from the coil, was constructed. This system is shown in Figure 2. Metal tubing was selected for the reaction coil since thin-walled Teflon tubing does not possess sufficient strength for safe operation under the conditions of temperature and pressure employed. Teflon tubing used initially in liquid baths, in the experiments with Freon mentioned above, and with silicone oil (Dow silicone oil DC-200, b.p. $> 200^\circ$), developed pinholes at 140° and 43 psig and created a

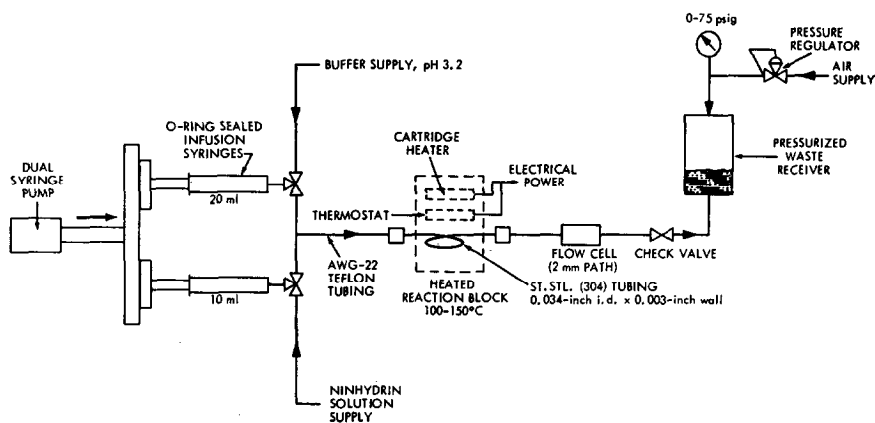


Fig. 2. Experimental arrangement for the high-pressure ninhydrin reaction.

hazard when the superheated water was released to atmospheric pressure. The use of thicker-walled Teflon tubing was rejected because of the reduced thermal conductivity through the wall.

Experimental Arrangement

Figure 2 shows the arrangement used to obtain color reaction temperatures up to 150° at pressures to 60 psig. Solutions of ninhydrin and buffer, the buffer containing additions of amino acids when desired to simulate effluent from a column, were mixed by feeding both into a common length of AWG-22 Teflon tubing from a variable-speed dual-syringe pump (Harvard Apparatus Co., model 943). In the configuration used in most of the work, the mixture was then led through a 24 in. length of 0.034 in. i.d. $\times$ 0.003 in. wall stainless-steel (304) tubing which was coiled and soft soldered to a $\frac{3}{4} \times 1 \times 2\frac{3}{4}$ in. copper block. A view of such a block (with 8 in. of tubing) is shown in Figure 3. The block was heated

by a cartridge heater (Vulcan Miniature, $\frac{3}{16}$ in. o.d., 10 W, 115 V AC) controlled by a cartridge thermostat (Vulcan Cal-Stat, model 1A1C2, $\frac{1}{2}$ in. o.d.).

The color reaction coil was connected via AWG-22 Teflon tubing to a flow cell (Pyrocell No. 5013, 2 mm path, 0.064 ml volume) held in a Beckman DB spectrophotometer in a mounting block designed in this laboratory. The flow cell was connected at both ends to Teflon adapters

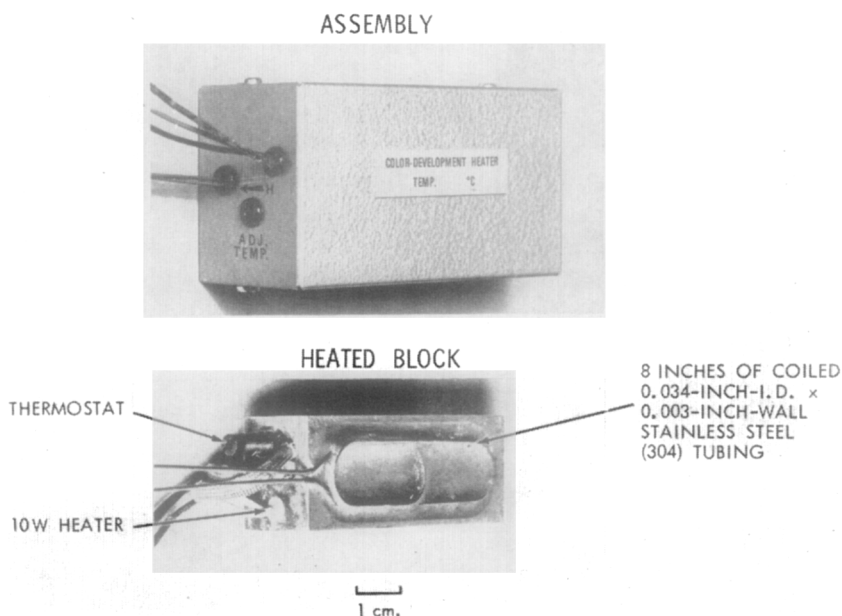


Fig. 3. Metal color-development heater assembly.

by O-rings. The Teflon tubing was connected to the adapters by treating mating surfaces with an etchant solution (Fluorocarbon Co., Teflon bonding kit No. 2), to allow sealing with a silicone adhesive (Dow Corning type 732 RTV). The discharge from the flow cell was received by an acrylic pressure chamber to which was applied an air pressure selected for system operation. A three-way valve (Hamilton Co., type 3NNN2) at the discharge of each pump syringe was used to refill the syringes (Becton-Dickinson No. 02-0010, 10 ml, and No. 02-0014, 20 ml) without exposure of solutions to air. Connections between the Teflon tubing from the syringes to the reaction coil and from the reaction coil to the flow cell were made with Swagelok union fittings (Crawford Fitting Co., type 100-6-316 with Teflon ferrules) by using a 0.042 in. i.d.

× 0.062 in. o.d. stainless-steel tube slipped over and silver soldered to the smaller metal tubing as an adapter sleeve.

Reagents

The ninhydrin reagent solution and the citrate buffer solution were made up in the manner prescribed in the manual for operation of the Beckman-Spinco amino acid analyzer. The formulations are based on those described by Moore *et al.* (10). The buffer is the pH 3.25 one commonly used as the first eluting buffer in the amino acid analyzer. Exact compositions for 1 liter of each solution are as follows.

Buffer: citric acid monohydrate, 21.0 gm; sodium hydroxide (97%), 8.25 gm; concentrated hydrochloric acid, 10.6 ml; caprylic acid, 0.1 ml; thiodiglycol, 5.0 ml; Brij 35, 2.0 ml.

Ninhydrin reagent: sodium acetate trihydrate, 136.0 gm; glacial acetic acid, 25.0 ml; methyl Cellosolve, 750 ml; ninhydrin, 20.0 gm; stannous chloride dihydrate, 0.4 gm. The pH of the sodium acetate/acetic acid mixture, diluted to 250 ml, was adjusted to 5.51 ± 0.03 before mixing with the Cellosolve and other ingredients.

For addition of amino acids, the Calbiochem (California Biochemical Corp.) No. 893001 standard mixture of 18 amino acids was used. L-Leucine and L-proline, which were added alone in some experiments, were products of Mann Biochemical Corporation.

Operation

A solution of the buffer containing either zero or 0.2 μ mole/ml total amino acid, made by diluting the standard mixture, was mixed with ninhydrin reagent at the 1.8:1 volumetric discharge ratio of the 20 and 10 ml syringes and then passed through the heated color reaction coil. Coil temperatures were selected in 10° increments from 100° to 150°, and the dwell time of the mixture in the heated coil section was determined

TABLE 1
Solution Dwell Periods at Selected Flow Rates

Buffer	Flow, ml/min		Dwell time, ^a min
	Ninhydrin	Total	
0.026	0.014	0.040	9.0
0.051	0.028	0.079	4.5
0.102	0.057	0.159	2.24
0.26	0.145	0.405	0.88
0.47	0.26	0.73	0.49

^a Assumed to be equivalent to the calculated passage time of a bubble through a 24 in. long heated section of the metal tubing.

TABLE 2
System Operating Pressures

Coil temperature, °C	Water vapor pressure, psig	Operating pressure, psig
100°	0	5 to 15 ^a
110	6.2	5 to 15 ^a
120	14.0	20
130	24.5	30
135	30.7	37
140	37.7	43
150	54.4	60

^a No differences in operation noted over pressure range.

by selecting the total flow rate from the pump, according to the values given in Table 1.

The air pressure applied in the liquid waste receiver was chosen about 6 psi above the vapor pressure of water at the reaction coil temperature to prevent boiling and to keep dissolved gases in solution (Table 2). The syringe pump was capable of discharge pressures up to about 70 psig

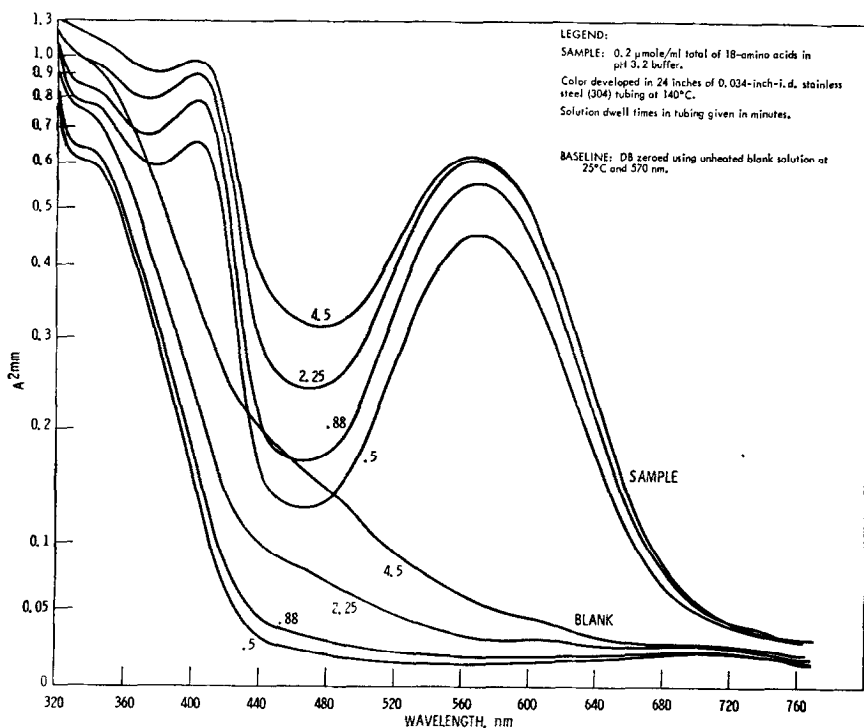


FIG. 4. Absorption spectra of ninhydrin reaction at 140° for increasing reaction periods.

without affecting the flow rate. No bubbles were observed in the Teflon tubing downstream of the reaction coil at the pressures employed, but bubbles were present whenever the air pressure was reduced to the water vapor pressure level.

A short length of the metal tubing immediately downstream of the coil was immersed in a 25° water bath to prevent further color development beyond the coil when the higher flow rates were used. At flow rates

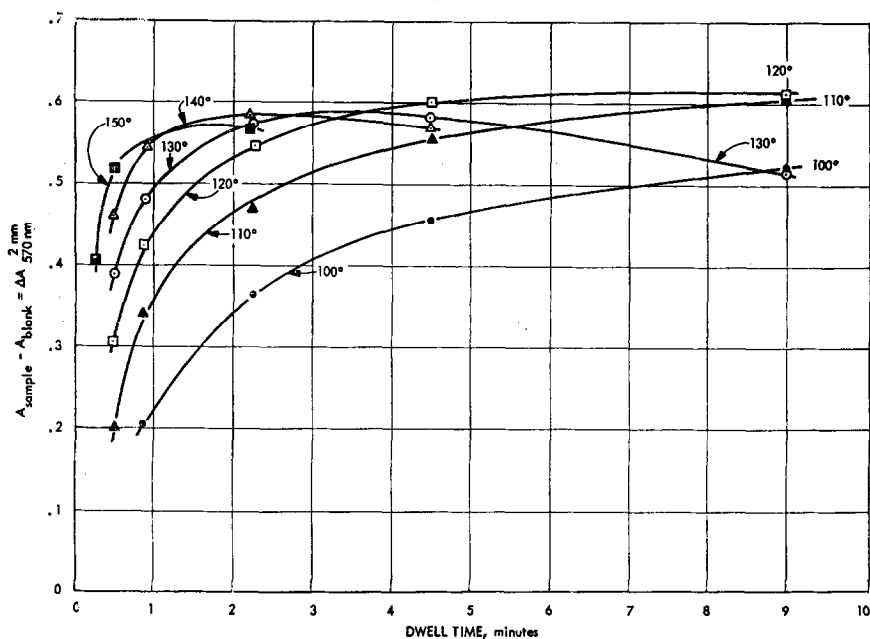


Fig. 5. Absorbance variation with dwell time at several temperatures.

below 0.2 ml/min, the tubing was cooled by the ambient air a short distance from the block. The flow from the coil was scanned from 320 to 760 nm for each set of operating conditions, with air in the reference beam of the spectrophotometer. The blank solution, containing no amino acid, was scanned under the same conditions.

RESULTS

Figure 4 shows absorption of the solution after exposure to a 140° reaction coil temperature for periods of from 0.5 to 4.5 min. Note that the absorbance of the solution without sample (blank) increases rapidly at the shorter wavelengths for the longer reaction periods and reaches an absorbance of 0.2 at 440 nm after 4.5 min. This rise is assumed to

result from a degradation of the ninhydrin in the reagent or from formation of a by-product. The tracings for the solution containing amino acid show that 90% of the color development at 570 nm is attained at the end of 0.88 min, a period during which no significant increase in absorbance has occurred in the blank solution at either 570 or 440 nm. Similar spectra were obtained with operation at the other coil temperatures listed in Table 2, the differences between these spectra being that

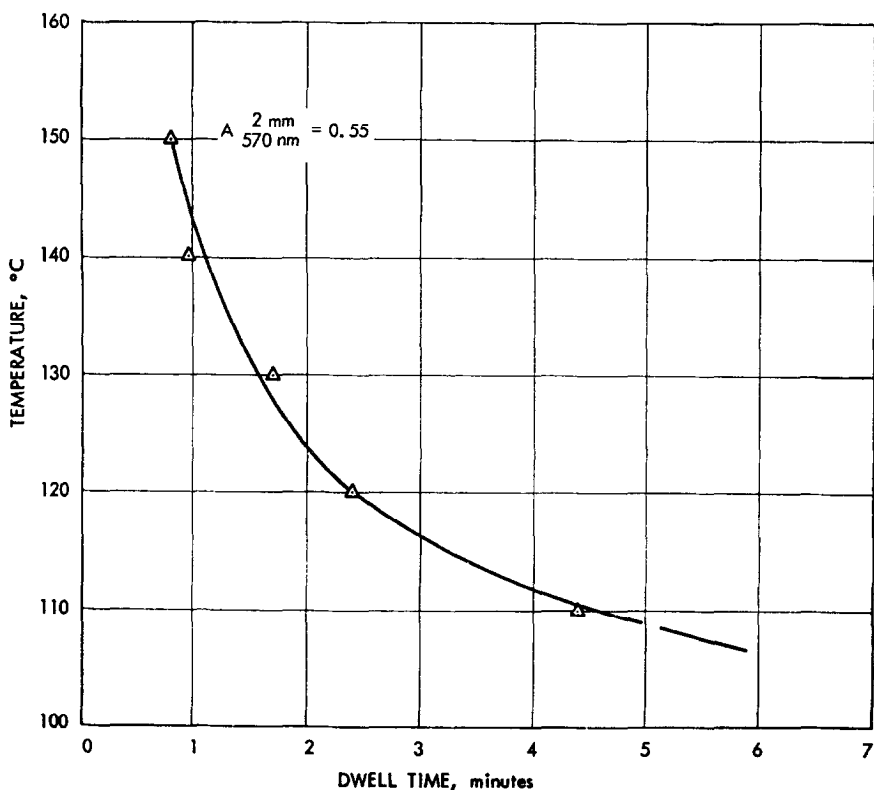


Fig. 6. Dwell time to produce an absorbance of 0.55 at several temperatures.

(a) only one-half the dwell time is required to produce the same absorbance for a 10° increase in coil temperature, and (b) the 470 nm valley rises as the coil temperature increases.

Figure 5 shows a plot of the absorbance increase (ΔA) produced by the reaction as a function of dwell time and coil temperatures. In all runs the absorbance of the blank solution at 570 nm was subtracted from that of the sample solution to eliminate baseline errors. It can be seen that the degree of color development reached in 9 min at 120° is greater

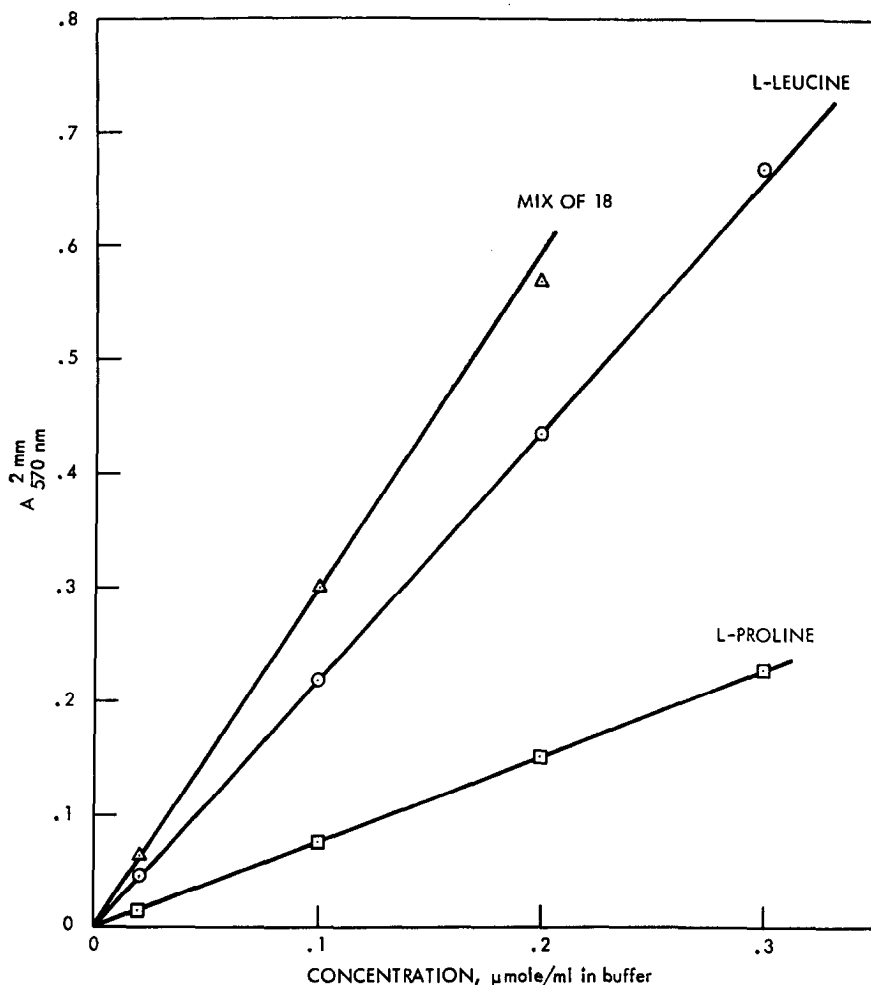


FIG. 7. Linearity of reaction at 135° for L-leucine, L-proline, and a mixture of amino acids. Absorbance of L-proline measured at 440 nm.

than at any other condition tested and that the response decreases with dwell time after the maximum is reached at the higher temperatures. This is presumably due to a partial breakdown of the reagent or products at the higher temperatures.

Figure 6 shows the dwell periods required to produce a somewhat lesser absorbance of 0.55 at the several temperatures. At this slightly reduced sensitivity, the color reaction can be completed in about 1.3 min at 135°. This temperature was selected for coil operation in the rest of the work as a compromise between a slightly shorter color develop-

ment time at 140°, with a resulting decrease in coil length of 1 in., and the higher pressure required at 140°. The 1.3 min dwell time could be attained with 8 in. of 0.034 in. i.d. $\times$ 0.003 in. wall tubing (allowing 1.25 in. of tubing for solution warmup) at a flow of 0.08 ml/min, which is the rate required to produce the same fluid velocity in a 3 mm diameter resin column as is obtained at 0.75 ml/min in a 9 mm diameter column. To check operation under these conditions, an 8 in. length of tubing was soft soldered on the block shown in Figure 3 and runs were made with the standard amino acid mixture. In addition, solutions of L-leucine and L-proline were each run to check linearity (Fig. 7) and to determine if operation at 135° significantly reduced the sensitivity of the color reaction. Figure 8 shows the spectra of the three solutions at 135° (solid lines) compared with the spectra from the reaction for 15 min at 100° in the same tubing (dashed lines). The pH of the solutions, measured at 25° following reaction, was 5.1.

The results for both the amino acid mixture and L-leucine at the two temperatures are essentially identical except that the 470 nm valley is

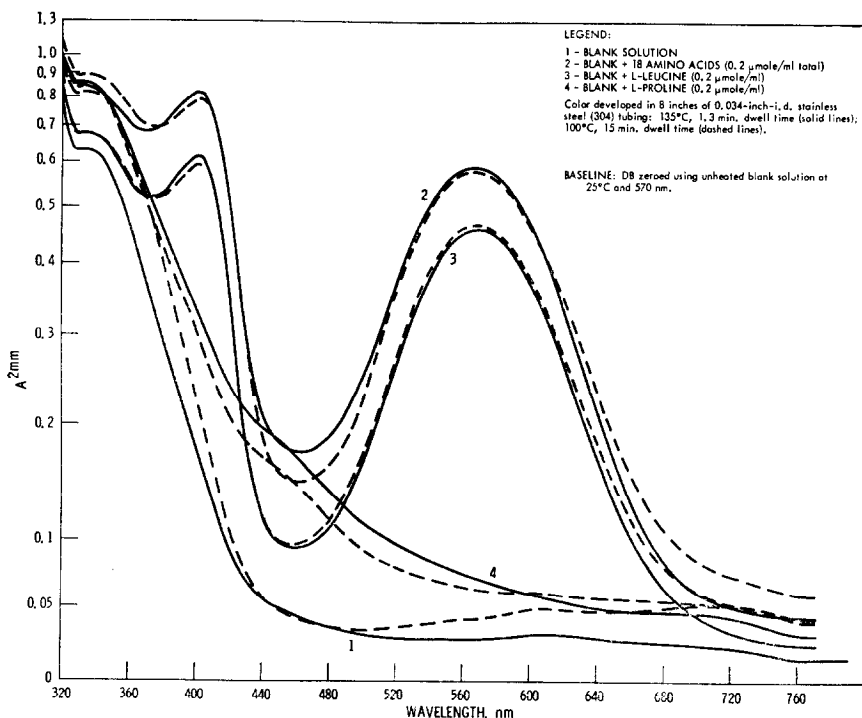


Fig. 8. Comparison of ninhydrin reaction at 135° for 1.3 min with reaction at 100° for 15 min for L-leucine, L-proline, and a mixture of 18 amino acids.

higher at 135° for the mixture. The sensitivity to L-proline (at 440 nm) increases about 25% at 135°. At coil temperatures above about 130°, a dwell time two to three times longer than required to reach maximum absorbance produces changes in the mixture and results in absorbance values (at 570 nm) greater than one, or precipitation of solid matter in the lines, or both. Frequently these solids will adhere to the flow cell windows and reduce the light transmission, but they can be easily redissolved by flowing a small amount of undiluted ninhydrin reagent through the system. For this reason, the tubing should be flushed with buffer solution to remove ninhydrin from the coil before flow is stopped.

A peak at 404 nm was observed in these experiments whenever color was developed at elevated temperature. It was not observed in the proline spectrum and was also missing when the 570 nm color was developed overnight at 25° by ninhydrin-positive substances extracted from several elastomeric stocks immersed in the ninhydrin solution.

As a test to determine whether the stainless-steel coil affected the color development, spectra were made of solutions withdrawn from the system before reaching the coil and then heated in a 100° water bath for 15 min. These scans were quite similar to those obtained from solutions passed through the metal coil at 100° with a 15 min dwell time.

In the event that the 0.034 in. tubing bore used here is too large for good chromatographic resolution in future work, it should be possible to replace it with about 11.5 in. of stainless-steel tubing having a bore equal to the nominal 0.027 in. i.d. of the AWG-22 Teflon tubing used elsewhere in this application.

DISCUSSION

These results indicate that the reaction time, and therefore the dimensions of the reaction train of the amino acid analyzer, can be very markedly reduced when the reaction is carried out at temperatures above the commonly used 100°. A temperature of 135° was chosen as a compromise among speed of color development, sensitivity, stability of the color formed, pressure, and length of reaction coil. The response of the system and the color stability compare favorably with the reaction as carried out at 100° with a longer dwell time. It is conceivable that the responses of the individual amino acids will differ slightly from the responses in the more commonly used system, in which case new constants will have to be determined.

While this report has been concerned mainly with study of some parameters of the ninhydrin reaction, the concepts of the system described can be applied to other analyzers using high-pressure separations with

feedback control and high temperatures and high-pressure reactions if necessary.

The system as described uses ninhydrin solution with reductant in it from the start, which necessitates protection of the solution from oxygen. At the price of some increase in the mechanical complexity of the injection apparatus, a stabilized ninhydrin reagent which is reduced only at the last moment before mixing with column effluent can be incorporated.

With the finding of satisfactory conditions for shortening the time of color development, the stepwise solution of the remaining problems connected with miniaturization of the amino acid analyzer as outlined may now be begun.

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