

buffer through the column, and the chromatograph tubes are equipped with semiball connections.

Columns of Dowex 50-X4 (9) also could be operated at faster flow rates in a similar manner. The separation of peptides on columns of Dowex 50-X2 (4, 7) can be speeded up by using a pump, which should be placed in the line between the reservoir bottle or mixing chamber and the column. Chromatograph tubes suitable for use with higher pressure (12) are then needed. The 2% cross-linked resin is available in a through-400-mesh grade (Technical Service and Development, Dow Chemical Co., Midland, Mich.) which will give good yields of material with small enough particle size to permit a flow rate of 30 ml. per hour per sq. cm. cross-sectional area of the column.

Allo Forms of Hydroxylysine and Isoleucine. Hamilton and Anderson (3) and Piez (11) observed that racemization at the α -carbon of hydroxylysine occurs during acid hydrolysis and gives rise to appreciable amounts of allohydroxylysine, which appears as a separate peak on effluent curves from columns of Dowex 50-X8. Piez (11) has also shown that alloisoleucine emerges ahead of isoleucine and at a position overlapping that of methionine from 100-cm. columns of Dowex 50-X8 (8). In the present method this overlap is eliminated (Figure 1), and allo-

isoleucine appears in a peak emerging between methionine and isoleucine. Any trace of alloisoleucine obtained as a result of racemization at the α -carbon of isoleucine during hydrolysis can thus be measured. When the fraction collector method is used, the amount of alloisoleucine in protein hydrolyzates is frequently too small to be detected. The recording method (12), however, is capable of demonstrating the trace of alloisoleucine present (up to 3%) after 24 hours of hydrolysis. After 72 hours of hydrolysis, the alloisoleucine formed may correspond to as much as 5% of the isoleucine, and the sum of both peaks probably should be used to calculate the isoleucine content of the protein, in the same manner that the methionine sulfoxides which may be formed during hydrolysis are included in calculating the methionine content of the protein.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Renate Mikk and Kerstin Johansson during these experiments. They are greatly indebted to P. B. Hamilton for sharing the details of his procedure during its developmental stages.

LITERATURE CITED

- (1) Crampton, C. F., Stein, W. H., Moore, S., *J. Biol. Chem.* **225**, 363 (1957).

- (2) Hamilton, P. B., *Anal. Chem.* **30**, 914 (1958).
- (3) Hamilton, P. B., Anderson, R. A., *J. Biol. Chem.* **213**, 249 (1955).
- (4) Hirs, C. H. W., Moore, S., Stein, W. H., *Ibid.* **219**, 623 (1956).
- (5) Hirs, C. H. W., Stein, W. H., Moore, S., *IUPAC Symposium on Protein Structure*, Wiley, New York, in press.
- (6) Mayer, S. W., Tompkins, E. R., *J. Am. Chem. Soc.* **69**, 2866 (1947).
- (7) Moore, S., Stein, W. H., *Advances in Protein Chem.* **11**, 191 (1956).
- (8) Moore, S., Stein, W. H., *J. Biol. Chem.* **192**, 663 (1951).
- (9) *Ibid.*, **211**, 893 (1954).
- (10) *Ibid.*, p. 907.
- (11) Piez, K. A., *Ibid.*, **207**, 77 (1954).
- (12) Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.* **30**, 1190 (1958).
- (13) Spackman, D. H., Stein, W. H., Moore, S., *Federation Proc.* **15**, 358 (1956).
- (14) Spedding, F. H., Fulmer, E. I., Butler, T. A., Gladrow, E. M., Gobush, M., Porter, P. E., Powell, J. E., Wright, J. M., *J. Am. Chem. Soc.* **69**, 2812 (1947).
- (15) Stein, W. H., *J. Biol. Chem.* **201**, 45 (1953).
- (16) Stein, W. H., Kunkel, H. G., Cole, R. D., Spackman, D. H., Moore, S., *Biochim. et Biophys. Acta* **24**, 640 (1957).
- (17) Stein, W. H., Moore, S., *J. Biol. Chem.* **211**, 915 (1954).
- (18) Tallan, H. H., Moore, S., Stein, W. H., *Ibid.*, **211**, 927 (1954).
- (19) *Ibid.*, **230**, 707 (1958).

RECEIVED for review February 28, 1958.
Accepted May 17, 1958.

Automatic Recording Apparatus for Use in the Chromatography of Amino Acids

DARREL H. SPACKMAN, WILLIAM H. STEIN, and STANFORD MOORE

The Rockefeller Institute for Medical Research, New York 21, N. Y.

► Quantitative determination of amino acids is made simpler and more rapid by an instrument for automatically recording the ninhydrin color value of the effluent from ion exchange columns. The influent buffer, freed of air, is pumped at a constant rate through a column of sulfonated polystyrene resin. The effluent is met by a capillary stream of ninhydrin reagent delivered by a second pump. The color is developed by passing the mixture of reagent and effluent through a spiral of capillary Teflon tubing immersed in a boiling water bath. The absorbance of the resulting solution is measured continuously at 570 and 440 μm as it flows through a cylindrical glass cell of 2-mm. bore. The peaks on the recorded curves can be inte-

grated with a precision of $100 \pm 3\%$ for loads from 0.1 to 3.0 μmoles of each amino acid. A hydrolyzate of a protein or peptide may be analyzed in less than 24 hours. The more complex mixtures characteristic of blood plasma, urine, and mammalian tissues can be analyzed in 2 days. The instrument is applicable in principle to detection of ninhydrin-positive constituents in the effluent from various types of chromatograph columns.

QUANTITATIVE determination of amino acids bears a relationship to the chemistry of proteins and complex peptides similar to that which elementary analysis bears to the chemistry of

simpler organic molecules. In recent studies on the structure of ribonuclease, a partial structural formula for the enzyme has been deduced primarily from data obtained by the quantitative amino acid analysis of the peptides formed as a result of the action of trypsin, peptidase, and chymotrypsin (4). Determinations of amino acids are also required in investigations of the distribution of amino acids and their derivatives in physiological fluids (16, 17) and mammalian tissues (18) as well as in the analysis of foods (10, 14). To facilitate such studies, there is need for an accurate method of amino acid analysis that is more rapid and less laborious than the techniques employed heretofore. To fill this need, a recording apparatus has been constructed which,

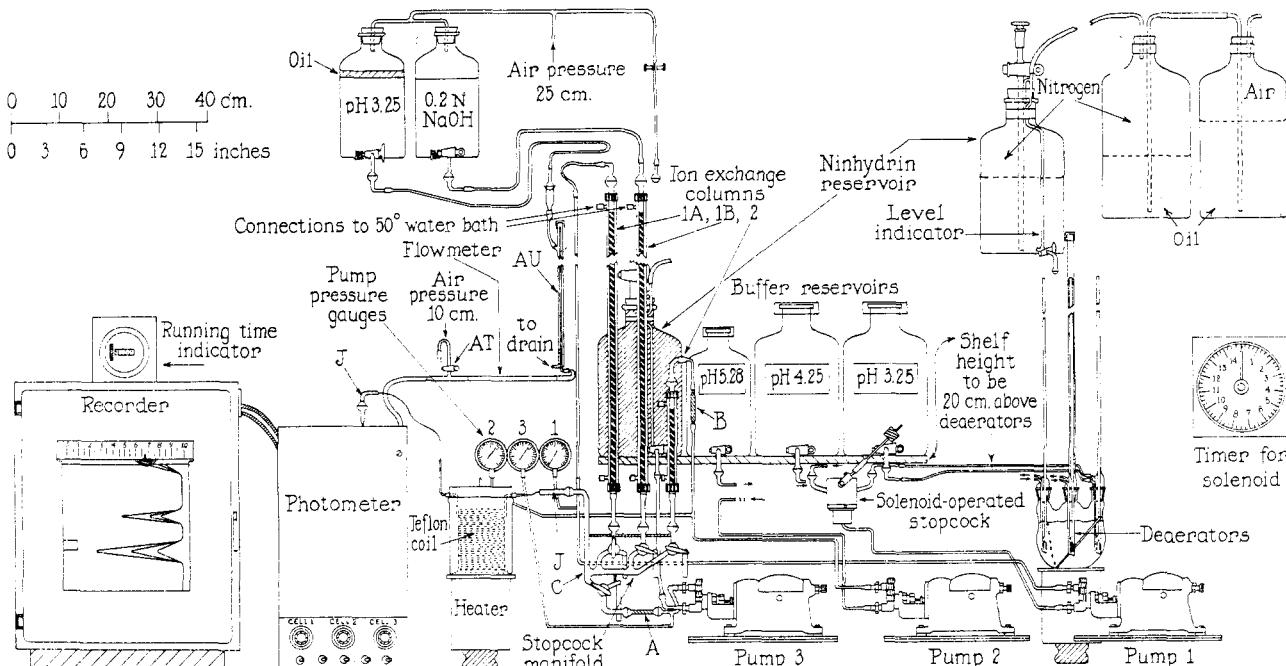


Figure 1. Automatic recording apparatus used in chromatographic analysis of mixtures of amino acids

Influent buffers are drawn from the reservoir bottles through the deaerating equipment and pumped through the ion exchange columns at a constant rate. Ninhydrin reagent is pumped into the effluent stream at C and the effluent-ninhydrin mixture is carried in a Teflon coil through a boiling water bath to develop the colors formed when ninhydrin reacts with amino acids. The absorbance of the colored solution is measured continuously by a photometer assembly and registered on a recorder. (See text for modification in arrangement of four bottles at the top of the figure, and use of a solenoid-operated valve instead of solenoid-operated stopcock.)

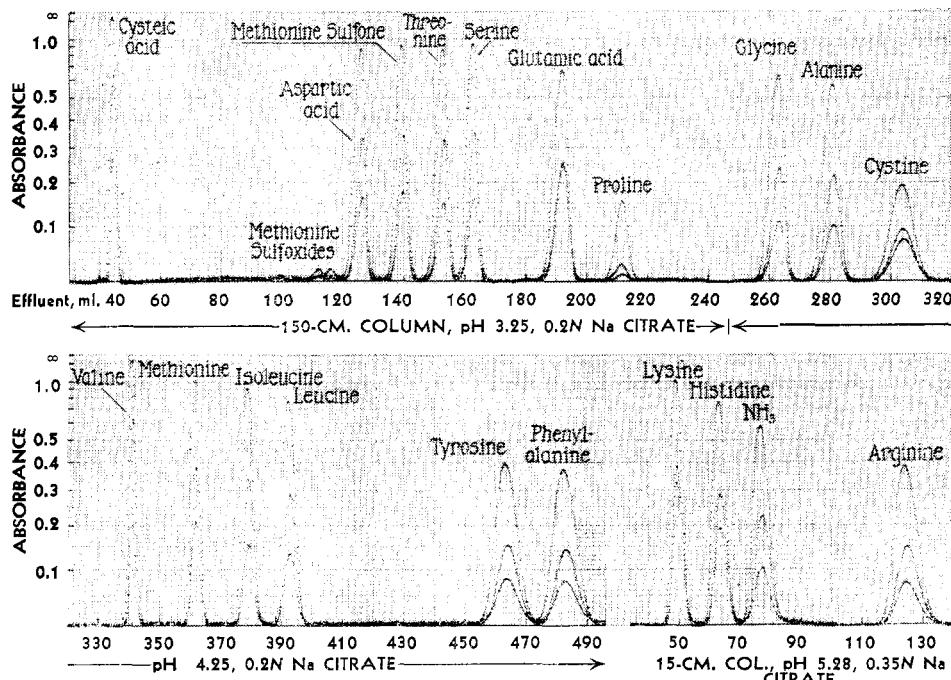


Figure 2. Chromatographic analysis of a mixture of amino acids automatically recorded in 22 hours by equipment shown in Figure 1

The three curves show absorbance at 570 m μ using two different cell depths and at 440 m μ for determination of proline. The chromatographic system is designed for the analysis of protein hydrolyzates (5). The method gives separate peaks for methionine sulfone, the methionine sulfoxides, cysteic acid, and S-carboxymethylcysteine (at 96 ml.). Hydroxyproline can be detected by the presence of a shoulder on the 440-m μ curve at the beginning of the emergence of aspartic acid; full separation of hydroxyproline requires the system illustrated in Figure 9A. Alloisoleucine, frequently formed in trace amounts from Isoleucine by racemization during hydrolysis of proteins, appears in a separate peak between methionine and isoleucine (5, Figure 1). Glucosamine and galactosamine follow phenylalanine from the 150-cm. column, and are eluted at 510 and 580 ml., respectively. The change to the pH 4.25 buffer can be delayed 1 hour, if elution of valine as a broader peak with the pH 3.25 buffer is preferred. Any residual tryptophan in an acid hydrolyzate emerges just ahead of lysine from the 15-cm. column; glucosamine, galactosamine, chlorotyrosine, and a ninhydrin-positive degradation product of tryptophan are detectable as constituents which emerge just ahead of tryptophan from the short column. Hydroxylsine and ornithine overlap tryptophan and lysine, respectively; their presence requires use of the system shown in Figure 9B.

in conjunction with improvements in ion exchange chromatography, permits the complete quantitative determination of amino acids in a hydrolyzate of a peptide or a protein every 24 hours with minimum attention.

In the ion exchange methods described previously (7, 8), the effluent from the column was collected as a large number of small fractions of known volume, in each of which the amino acid content was determined individually by a quantitative photometric ninhydrin method (6, 9). In the present automatic technique (15), ninhydrin reagent is introduced at a constant rate into the effluent flowing from the column. Color development ensues as the mixture is pumped through a coil of capillary tubing immersed in a boiling water bath, after which the intensity of the blue color (diketohydridene-diketohydridamine) formed when amino acids are present is determined by continuous photometry at 570 m μ . The yellow colors from proline and hydroxyproline are also monitored at 440 m μ . The results are plotted automatically on a recording microammeter. A schematic drawing of the apparatus is shown in Figure 1, and an effluent curve is shown in Figure 2.

APPARATUS

Pumps. To render a continuous method precise and reproducible, both the eluent and the ninhydrin reagent must be delivered at constant and controllable rates by reliable pumps.

The instrument illustrated in Figure 1 uses three small, piston-type pumps of stainless steel, each of which has two ball check valves in series on both inlet and outlet lines (Milton Roy Co., 1300 East Mermaid Lane, Philadelphia 18, Pa.). The step-valve type pumps which were used initially were specially selected for better than 90% efficiency. The manufacturers have more recently placed on the market a smaller column-valve which they recommend for this application. The specifications are: Minipump MM1-B-29 for chromatographic applications, range 0 to 320 ml. per hour, factory-tested for efficiency of delivery, column valve and seats of Carpenter 20 SS, with ball checks and $\frac{1}{8}$ -inch plunger of Hastelloy C, black Teflon packing, and Bodine motor (Catalog No. B4210-60H) $\frac{1}{15}$ hp., Type NSI-34RH, gear-reduced to 29 r.p.m. The two $\frac{1}{8}$ -inch pipe thread fittings connecting the inlet and outlet tubing to the column-valve are Swagelok tubing connectors (elbows) of 316 SS (Part 300-2-2-316, Crawford Fitting Co., 884 East 140 St., Cleveland 10, Ohio). These pressure-tight connectors are now preferred to the hose nipples illustrated in Figure 1; the fittings are tightly threaded using ordinary pipe thread compound.

Two pumps deliver buffer to the columns at 30 ml. per hour; the third supplies the ninhydrin reagent at 15 ml. per hour. Delivery is regulated by a screw and vernier adjustment which controls the length of the piston stroke.

To achieve satisfactorily constant delivery, at the low flow rates employed, it has usually been necessary to reduce the end play between the base of the piston and the crosshead by removing the nut which couples the piston to the crosshead and making this nut slightly shorter by the use of fine emery cloth. The final adjustment should still permit the piston to be easily rotated between the fingers when the retaining nut is tightened and the base of the piston is lightly oiled. The plunger is then still loose enough for self-alignment. If the nut is too short, the flat side of the washer which bears against the base of the piston can be similarly treated to restore freedom of movement. Hot detergent solution should be flushed through each pump with suction before it is installed.

The Teflon packing is assembled, air-free, in glycerol. With the valve removed from the frame, the outlet and inlet are closed, and the well in which the packing fits is filled with glycerol. Each piece of packing is inserted in proper order. The packing gland follower is placed on top and pressed in gently before the valve is attached in operating position on the pump. The packing nut should be set only finger-tight.

A new pump, or one into which air has been accidentally introduced, should be operated at full stroke against no

back-pressure for a few minutes. Momentary application of slight suction may also be required to dislodge air bubbles. In the case of pump 2 or 3, the outlet line must be opened at the ball joint just preceding the pressure filter (*A* or *B*, Figure 1) before the plunger is driven at full stroke. Care must be taken not to empty the corresponding deaerator, and thus to draw in more air. The pump is then set to deliver at nearly the desired rate (about 5 or 10% of full stroke).

The principal cause of variable delivery in a used pump is accidental introduction of air from the lines or through wear of the plunger and the Teflon packing. If leak-free performance is not obtained when the packing nut is finger-tight, the packing probably needs replacement. If the shaft of the plunger is detectably scored, a new one should be installed. When these operations do not lead to steady performance, the ball checks and seats should be examined for wear. S.A.E. 60 oil is used in the lubricating bath; oil reaching the outer cup should not be re-used. The coupling from the motor shaft is pressed close to the pump housing, to minimize leakage of oil from the bearing.

The pressure in each outlet line is registered on a 0- to 60-pounds per square inch gage (Industrial Gauge and Instrument Co., Inc., 1947 Broadway, New York 23, N.Y., Figure 500S, 2 inches in diameter, 60-pound $\frac{1}{8}$ -inch L.M.). The connection is made by Swagelok Part 300-7-2-316. The tee in the line can be of glass (2-mm. bore) or metal (Swagelok part No. 300-3-316), and is positioned so that air bubbles will not enter the gage line. Initially, before connection, the gage and the attached tubing (at least 50 cm. long) are evacuated on a water pump and allowed to fill with water; air in the gage slows the rate of attainment of equilibrium pressure in the system.

Buffer and Reagent Reservoirs. The reservoirs for the buffers are made from 2- and 4-liter aspirator bottles (Corning No. 1220) by attaching a 2-mm. bore stopcock to the outlet. A 12/5 ball joint with a 90° bend is attached to the stopcock (Figure 1), with a 12/2 socket joint leading to the flexible tubing. The bottles should be mounted 20 cm. above the outlet tubes of the deaerators, to provide positive pressure to the deaerators and, in turn, to the pumps, for the satisfactory operation of which a pressure of at least 30 cm. of water on the inlet lines is desirable. A drying tube filled with powdered citric acid can be used at the top of the reservoir containing the pH 5.28 buffer to prevent uptake of ammonia from the air. Bottles should not be directly over any electrical parts of the instrument.

The flexible tubing on all of the buffer lines is Tygon, B 44-3, $\frac{1}{16}$ -inch (inside diameter) and $\frac{3}{16}$ -inch (outside di-

ameter). The slip-on connections to glass tubing are made with Tygon warmed in boiling water; the cooled tubing is wired in position. The two sizes of ball and socket joints (12 and 18 mm.) are held with appropriate spring clips (Arthur H. Thomas Co., Philadelphia 5, Pa.). Leak-proof performance from these joints is most readily obtained by insertion of silicone rubber washers (suggested by F. M. Richards). The washers are cut with cork borers from thin sheeting (Grade MD 50 silicone rubber, $\frac{1}{64}$ inch thick, Rway Synthetic Products Division, Reiss Manufacturing Corp., Little Falls, N.J.). Three sizes of washers have been used, respectively, for Corning 18/9, 12/5 or 12/2, and 12/1 ball and socket joints: cork borers 6 and 11, $\frac{3}{8}$ -inch (inside diameter) $\times \frac{5}{8}$ -inch (outside diameter), 5 and 8 ($\frac{5}{16} \times \frac{7}{16}$ inch), and 1 and 3 ($\frac{1}{8} \times \frac{7}{32}$ inch).

The ninhydrin reservoir is also made from a 4-liter aspirator bottle. It has a 2-mm. bore stopcock and an 18/2 ball joint sealed to the outlet; a vertical length of graduated 2-mm. bore capillary tubing joins the outlet with the nitrogen inlet tube (by flexible connections) to serve as a level indicator (Figure 1). A level indicator is necessary, because the reservoir is completely wrapped with black Scotch electrical tape No. 33 to protect the reagent solution from light. The top of the ninhydrin reservoir is closed by a rubber stopper, through which are passed a short length of glass tubing (for the nitrogen connection) and a longer tube (8-mm. inside diameter) extending nearly to the bottom of the reservoir. A 4-mm. bore stopcock is attached to the top of this long tube, and above the stopcock is a $\frac{3}{4}$ 14/35 outer joint. The ninhydrin reagent is stored under nitrogen (6), but the additional bottles (Figure 1) contain mineral oil rather than water. Instead of the bottles shown in Figure 1, two aspirator bottles (Corning No. 90355, $2\frac{1}{2}$ gallons) should be used, and the connection made at the bottom with $\frac{3}{8}$ -inch Tygon tubing (inside diameter).

The line from the ninhydrin reservoir to the pump starts with a 10-cm. section of 5-mm. bore tubing with an 18/7 socket joint on one end and a 12/2 ball joint on the other; this tube is loosely packed with borosilicate glass wool, to filter out any suspended particles in the reagent solution. Water should flow through the tube readily under gravity; if the packing is too tight, the inflow to the pump will be insufficient. When the entire section of glass wool becomes discolored, the filter is removed and regenerated by first drawing water and acetone through with suction. Cleaning solution is drawn into the thoroughly air-dried tube and allowed to remain for about an hour. The completely rinsed tube is replaced filled with water. Teflon tubing ($\frac{1}{8}$ -inch inside diameter, $\frac{3}{16}$ -inch outside diameter from Pennsylvania Fluorocarbon Co., Inc., 1115 North 38th St., Philadelphia 4, Pa.)

is used for the flexible connections to and from pump 3 and gage 3. Silicone rubber washers are preferable to grease in all the ball joints on the ninhydrin line.

The two 2-liter bottles for 0.2*N* sodium hydroxide and pH 3.25 buffer (top of Figure 1) are preferably plain bottles with siphon tubes entering from the top, rather than the aspirator bottles illustrated. The stoppers are wired in; 12/5 socket joints on the air inlet tube and on the siphon tube permit the reservoir to be readily disengaged when filling is necessary. The lines to the columns are of Tygon (R-3603, $\frac{1}{16}$ -inch inside diameter, $\frac{1}{8}$ -inch outside diameter). Stopcocks or pinch clamps are used near the end of each line; a 1-mm. bore Teflon and glass stopcock (Fischer and Porter Co.) may be employed for the sodium hydroxide. A drain cup attached to the top of *AU* (Figure 1) is conveniently located near the tops of the columns. In addition, a vacuum line attached to a trap is useful for withdrawal of buffer and sodium hydroxide from above the resin in the columns.

Deaerators. The buffer from each reservoir passes first through a deaerator, to remove dissolved air that would otherwise come out of solution as bubbles during passage of the buffer-ninhydrin reagent mixture through the heating coil in the 100° bath. Each deaerator is made according to the design in Figure 3 (Catalog No. 570523, Scientific Glass Apparatus Co., Inc., Bloomfield, N. J., to be supplied without the glass wool). About 25 mg. of borosilicate glass wool is inserted, by using a wooden applicator stick or wire, and should be packed tightly enough to cause air to be liberated to the extension tube in a stream of fine bubbles. Too loose a packing allows the formation of large bubbles that may block the inflow line or may be drawn into the outlet line leading to the pump (air from this source is the most likely cause of temporary pump failure); too tight a packing unduly restricts the flow from the reservoir. Each deaerator should be checked for leaks before installation; if detergent leaks into the boiling water bath, excessive foaming occurs.

In Figure 1, a separate boiling water bath is illustrated for the deaerators, but it is simpler to use a single bath to contain both the heating coil and the deaerators.

The bath consists of a 1.5-liter "resin reaction" flask [Scientific Glass Apparatus Co., Inc., bottom section, 10 inches high, Catalog No. J-1300-2, with a top section, specially ordered, carrying four $\frac{1}{2}$ 24/40 joints and one $\frac{1}{2}$ 14/35 joint, clamp No. J-1300-11, and Glas-col heating mantle H-1930 designed for a 1-liter (not 1.5-liter) flask]. The small joint carries a short connecting tube with a $\frac{1}{2}$ 14/35 joint on each end and two horizontal tubes sealed on for the entrance and exit of the heating coil tubing. A 65-cm.

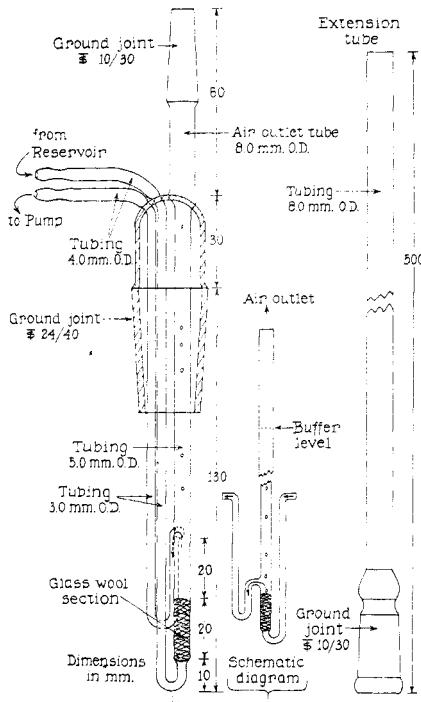


Figure 3. Deaerator

Dissolved air in influent buffers is discharged through extension tube; glass wool section is maintained at 100° C. (cf. Figure 1)

air condenser extends from the top of this connecting tube. High vacuum silicone grease is used on the joints and on the flange of the reaction flask. The fourth $\frac{1}{2}$ 24/40 port, normally stoppered, accommodates a fourth deaerator if the apparatus is to be used interchangeably for the analysis of protein hydrolyzates and physiological fluids. A boiling stick (with an air cup at the bottom) is inserted through the center port. A variable transformer is used to adjust the rate of boiling so that condensation occurs about 15 cm. from the top of the air condenser. The heat is normally left on continuously.

Solenoid-Operated Valve. The buffer of pH 5.28 goes directly from the deaerator to pump 2, but the other two buffers pass through a three-way solenoid-operated microvalve (Catalog No. 198, Barworth, Inc., Summit, N.J.) before reaching pump 1. This commercially available device, designed to replace the specially built solenoid-operated stopcock shown in Figure 1, permits the change of eluent required in the operation of the 150-cm. column (5) to be made automatically at any preset time. The body of the valve is of 316 stainless steel. The solenoid is energized from the white outlet of the Gra-Lab timer (Model 173, Dimco-Gray Co., 207 East Sixth St., Dayton 2, Ohio). The buffer at pH 3.25 enters the normally open port of the valve.

Pressure Regulators. Optimum performance of the pumps requires a back-pressure on the outlet valves.

The 150-cm. column supplies the

necessary pressure for pump 1, but additional resistance in the line is desirable for pumps 2 and 3. This has been obtained by inserting two glass wool filters at *A* and *B* in Figure 1. *B* is an 8-cm. length of 2-mm. bore tubing with 12/2 ball and socket joints at each end. The glass wool is packed tightly enough to give a back-pressure of about 25 pounds, which, combined with the resistance of the short column and the heating coil, gives operating pressures of 35 to 40 pounds. The pressure filter tube on the ninhydrin line, *A*, consists of a 5-cm. length of 2-mm. bore tubing (12/2 ball) sealed to a 1-cm. section 5 mm. in bore (12/5 socket). The glass wool is packed to give a pressure of 20 pounds. The wider segment near the pump collects some insoluble material that passes the loose filter on the inlet line. A spare pressure filter for the ninhydrin line is kept on hand to replace the used one when the pressure rises 10 to 15 pounds above the initial value. After removal, the filter is cleaned as described; it is usually necessary to let it remain overnight in cleaning solution.

An air pressure of 5 pounds is used during addition of samples to the columns and during washing and re-equilibration of the resin. This is supplied from the laboratory compressed air line by a Nullmatic regulator [(7), footnote 3, and (8), footnote 3], with either a 0- to 15-p.s.i. gage or a 0- to 30-cm. of mercury gage.

Chromatograph Tubes. Heavy-walled, jacketed chromatograph tubes, 0.9 cm. in diameter (7, 8), are modified for use in this apparatus. An 18/9 socket joint is ground onto the top of the long tube to give a length of 166 cm. from the top of the joint to the sintered plate, which should be of medium porosity and 3 mm. thick. A plate thinner than 2 mm. may permit some leakage of resin particles into the effluent lines and has a shorter life; a very slow deterioration is caused by the 0.2*N* sodium hydroxide, used to clean the columns. The diameter of the tube is narrowed to 1 mm. as close as possible beneath the plate, and the tube ends in a 12/1 ball joint. The shorter tube is constructed similarly, with a length of 25 cm. above the sintered plate. Tubes of this design, with water jackets to fit, are available from the Scientific Glass Apparatus Co., Inc. (Catalog No. SJ-1665-1-B, heights to be specified). If physiological fluids are to be analyzed, a tube with a height of 60 cm. above the sintered plate is also required.

The buffer line from pump 1 to the top of the column may include a section of 1-mm. bore glass tubing (Figure 1), to reduce the amount of flexible tubing on this pressure line. The connection to the top of the long column is made with an 18/1 ball joint (tubing 7 to 8 mm. in outside diameter with 1-mm. bore, Scientific glass Apparatus Co., Inc.) and a silicone rubber washer. Two spring clips placed opposite one another should hold the joint leak-free.

at pressures up to 70 pounds. The glass shoulder on the upper joint should be rounded enough to spread the arms of the first clip so that they are parallel when the clip is all the way on the joint; the second clip is slipped just over the ends of the first.

The joints held with spring clips also provide important safety valves in the system; a spring clip connection is present between each pump and the first source of resistance on the outlet line. In the event of any stoppage in the line, a slow leak will develop at this connection before any dangerous pressure can be built up.

Stopcock Manifold. The three columns and the ninhydrin pump are connected to a series of stopcocks joined by 1-mm. bore capillary tubing, as illustrated in Figure 4 (Scientific Glass Apparatus Co., Inc., Catalog No. 550916). If the equipment is to be used interchangeably for the analysis of protein hydrolyzates and physiological fluids, a manifold with a fourth stopcock in the upper row should be obtained. The stopcocks have solid plugs and are individually ground with maximum precision. By the use of the three-way, 120° stopcocks, the effluent from each column can be directed either into the heating coil or to waste. Ninhydrin reagent enters through the bottom stopcock, and can go to drain or be injected into the column eluate at C, Figure 1.

The stopcocks are lubricated with a minimum of Dow-Corning high vacuum silicone grease. Every few months the stopcocks are thoroughly cleaned with benzene and regreased. To prevent leakage under the back-pressure of about 7 pounds that prevails at this point in the system, the plugs of the stopcocks are held in place by stainless steel spring clips (stopcock tension clips, size 16, Todd Scientific Co., 160 State Road, Springfield, Pa.). The springs of the clips should be initially stretched so that they maintain a length of 5 cm., thus increasing the tension applied. All stopcocks should be checked for leakage by observing whether there is at any time escape of fluid from the drain tip when the stopcock is being used in the recorder position. The barrels and plugs of the stopcocks are marked so that they can be accurately positioned.

To minimize mixing in the effluent stream, all lines between the columns and the photometer should be a 1-mm. bore or less. If the tops of the columns are inconveniently high, the columns can be lowered nearly to floor level and connected to the manifold (at bench height) by capillary Teflon tubing joined to the glass as described below for the heating coil. A minimum of grease is usually sufficient to permit leak-free connections at the 1-mm. ball and socket joints on the effluent (low pressure) side of the equipment. Silicone rubber washers, centered on the lightly greased joints, are

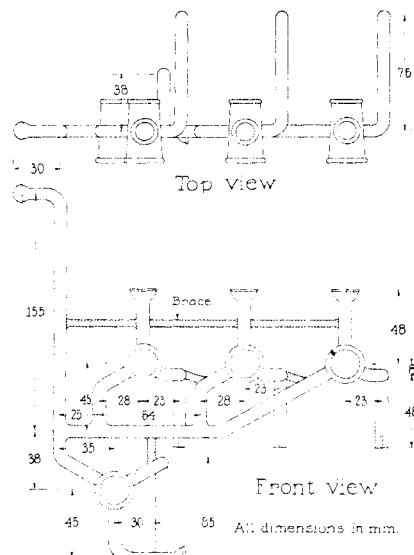


Figure 4. Stopcock manifold

All tubing is 1-mm. bore. The stopcocks are 3-way, 120°, 1-mm. bore, individually ground. A fourth stopcock in the upper row is required if the instrument is to be used interchangeably for analysis of protein hydrolyzates and physiological fluids.

used here only if tight connections cannot otherwise be obtained.

Heating Coil. The mixture of effluent and ninhydrin reagent from the manifold flows through a coil of flexible tubing 0.7 mm. in bore and about 95 feet in length, immersed in the boiling water bath. The coil is made with Teflon tubing, AWG size 22, nominal 0.027-inch inside diameter, wall thickness 0.012 inch (Pennsylvania Fluorocarbon Co.; specify a continuous length of 110 feet). As the Teflon tubing may vary slightly in internal diameter from lot to lot, the volume per unit length may be determined by weighing the coil of tubing both empty and full of water. This information is used to calculate the length of tubing (about 2900 cm.) which would contain 11.25 ml., thus allowing the effluent-ninhydrin solution mixture flowing at a total rate of 45 ml. per hour to remain in the boiling water bath for 15 minutes, sufficient time for maximal color development from most amino acids (9).

The necessary length of tubing is wrapped around a section of brass tubing 3 inches in diameter and 7 inches long (wall thickness $\frac{1}{16}$ inch). An extra 4 feet of Teflon tubing is left on the outlet end and an extra 2 feet on the inlet end. Four to eight short pieces of $\frac{1}{8}$ -inch copper or silver rod are soldered radially at the top and at the bottom of the brass cylinder to center it in the bath. Eyelets of copper wire are soldered near the top rim and at the bottom to hold the first and last loops of Teflon tubing in position. Two vertical brass tubes ($\frac{1}{8}$ inch) are attached to the cylinder to guide the ingoing and outgoing sections of Teflon tubing to a position just below $\frac{1}{4}$

14/35 port in the glass cover. The extra lengths of tubing at each end are brought out of the bath through the adapter inserted in the $\frac{1}{4}$ /35 joint leading to the air condenser. The points of emergence of the Teflon tubing from the two horizontal tubes of the adapter must be made vapor-tight by a micro one-hole rubber stopper (Arthur H. Thomas Co., No. 8823 E), or by reducing the ends of the glass tubes to 1-mm. bore and using a waterproof cement.

The connections (J, Figure 1) between the Teflon tubing and both the stopcock manifold and the absorption cell are made as follows:

The plastic tubing is threaded through a 3- to 6-cm. length of the 1.5-mm. bore tubing attached to a Corning 12/1½ ball or socket joint. The end of the Teflon tube protruding through the joint is flared slightly by the use of the tapered end of a 2-mm. glass rod that has been warmed in a flame. With the flared end extending a few millimeters through the joint, a drop of Chemiseal 201 adhesive (Plastics Division, United States Gasket Co., Camden 1, N. J.) is added at the other end and drawn down around the Teflon by suction until the adhesive is within 1 to 2 mm. of the ground joint. The Teflon tubing is then pulled back until the flare fits flush with the end of the ball or socket. The connection is left overnight in a horizontal position to allow the cement to set.

Photometer. From the bath, the liquid flows to the photometer (Figure 5). In the most recent instruments, the photometer assembly has been combined with the timers in one housing.

This housing has an aluminum frame upon which aluminum sides are fastened, a construction that gives a lighter and more rigid assembly than the $\frac{1}{4}$ -inch Bakelite housing shown in Figure 5. The partition corresponding to AK in Figure 5 is of $\frac{1}{4}$ -inch aluminum and extends all the way to the top of the frame. Both the lamp assembly and the absorption cell housing block are mounted on this partition plate rather than on the sides. All sheet aluminum surfaces inside the cabinet are finished in flat black to minimize reflections of light from the exciter lamps. The Gra-Lab timer is removed from its housing for panel mounting in the photometer.

The photometers have been constructed in the Instrument Shop at the Rockefeller Institute, but wherever possible, commercially available parts have been used.

The assembly consists of three individual photometer units. Each unit has a light source, K, a lens, L, an interference filter, M or N, and a photo-voltaic cell, O. The absorption cell,

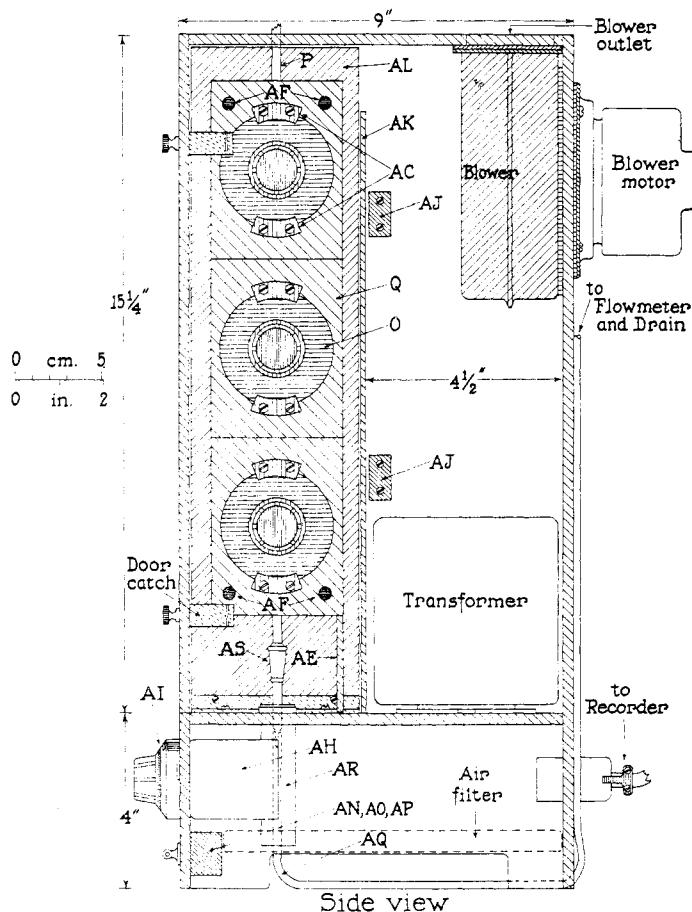
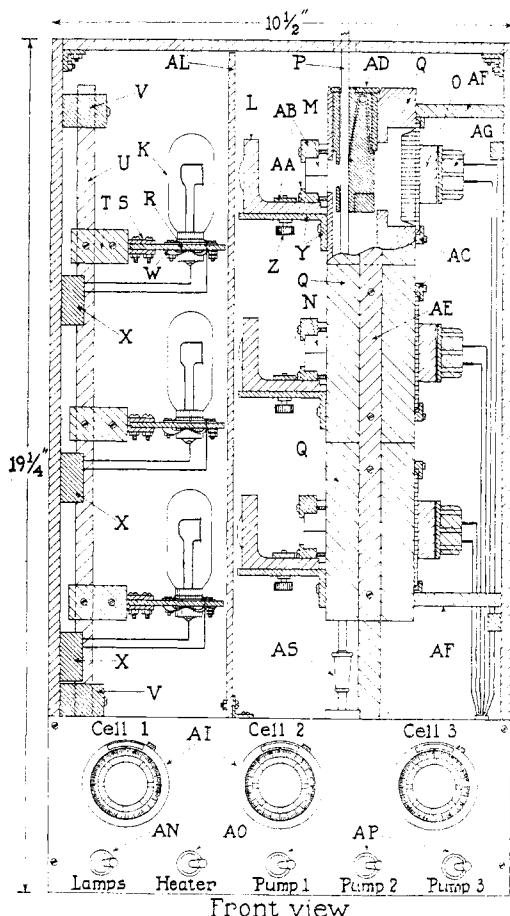
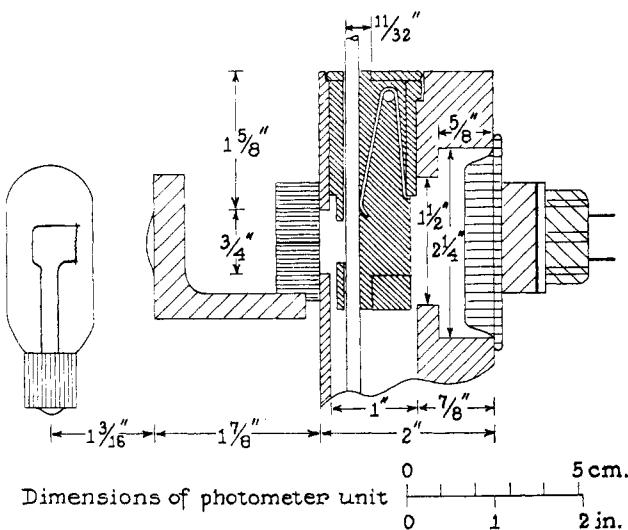
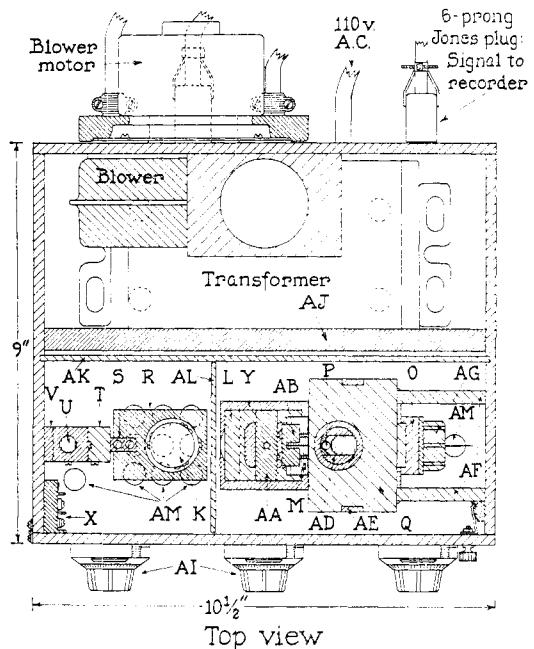


Figure 5. Photometer assembly

K, exciter lamps (No. 6-500, Coleman Instruments, Inc., Maywood, Ill.); L, lens assembly (Technicon Chromatography Corp., New York 51, N.Y., parts A 15, A 16, A 17, and A 18, focal length 3/4 inch, diameter 0.975 inch, thickness at center 0.375 inch, biconvex); M and N, interference filters, 570 and 440 m μ , respectively; O, General Electric photovoltaic cell PV2FAA; P, absorption cell; Q, absorption cell housing block in 3 parts; R, exciter lamp support plate; S, aluminum rod, that can turn in block T; U, vertical support rod held in blocks V; W, crimped spring washers, or a prefocus base (Morse No. 31 socket) with hand-made springs of double strength; X, insulated connecting blocks for 6.3-volt leads to lamps; Y, lens support, grooved to keep sliding lens centered over slit in adapter; Z and AA, thumbscrew and plate fastener for lens assembly; AB and AC, clamps for filters and photocells; AD, absorption cell adapters (adapter 6-110 for Coleman Junior Spectrophotometer, Coleman Instruments, Inc.); AE, strips holding the three housing blocks together; AF, housing block mountings; AG, socket, 8-prong; AH, Helipot, 1000-ohm (Model A R1K L5, Helipot Corp., Newport Beach, Calif.); AI, turns-counting dials (Duodial, Model RB, Helipot Corp.); AJ, cross braces; AK, partition; AL, partition, with 7/8-inch holes opposite each lens; AM, ventilation holes, 1/2-inch diameter; AN, AO, and AP, toggle switches; AQ, plastic drain tubing, Tygon, 1/16-inch inside diameter; AR, glass sleeve; AS, ground joint, $\frac{5}{12}$, clamped together.

P, containing the flowing liquid traverses all three units, and is mounted in the vertical aluminum block, *Q*. Three separate photometer units are necessary to provide complete data from a single analysis. The interference filters of the upper and lower units transmit maximally at 570 m μ , whereas the filter of the middle unit transmits maximally at 440 m μ . The upper photometer unit, in which the internal diameter of the absorption cell is 2.2 mm., measures the concentration of the blue compound formed in the reaction of ninhydrin with amino compounds (6). The second unit, in which the absorption cell also has a light path of 2.2 mm., is used only to measure the yellow color formed from proline or hydroxyproline, when present. The third unit measures the absorption at 570 m μ , but the absorption cell at this point is effectively thinner. Because of the decreased thickness of solution through which the light must pass, concentrations of blue color too great for determination in the upper unit can be estimated accurately, and the range of the instrument is thereby extended.

The type of mountings used for the lamps permits them to be moved freely in order to obtain the sharpest focus. The electrical leads to these lamps are carried up the side of the instrument and held in place by insulated mountings, *X*. Both of the wires to the lamps are soldered directly to the base of the lamp; reliance on any press contact through the lamp socket can lead to variable performance. To increase the life of the exciter lamps, they are operated at 6.3 volts instead of their rated 8.5 volts (4.2 amperes).

When the instrument is first set up, and before it is standardized with a known mixture of amino acids, a record should be taken of the potentiometer setting required to zero the recorder when ninhydrin reagent and buffer are passing through the system. A bulb should be replaced if the filament is giving a variable response (as evidenced by sudden changes in readings from a given photometer unit) or when the reading on the potentiometer required to zero the instrument reaches a value 300 ohms above the initial reference setting. Under these conditions, the life of the lamps is about 12 months for the units recording at 570 m μ , and about 6 months for the bulb used with cell 2 at 440 m μ . If new bulbs require a setting that differs by more than 100 ohms from the initial resistance used with the previous lamps, the instrument should be re-standardized.

The lens is attached to the absorption cell housing block, *Q*, in a position which centers it in front of the slit in the adapter, *AD*. The initial focusing of the system is made with the filter removed and with a small piece of white paper placed on the slit so that the centering of the light on the slit can be observed. The lens can be moved

by loosening the thumbscrew, *Z*, and sliding the lens forward or backward in the groove in the lens support, *Y*. With the paper removed and the filter in place, first the lamp and then the lens are adjusted until maximum current is elicited from the photocell. Once the proper adjustments of both the lamps and the lens have been secured, refocusing is seldom necessary, even when lamps are changed.

The filters in the photometer units must transmit as nearly monochromatic light as possible, and must permit passage of enough incident illumination to cause the photovoltaic cells to generate at least 10 μ a. for operation of the recorder. Very satisfactory results were obtained with multilayer dielectric-type interference filters (1 inch square) having a transmittance of about 70% of the incident light at their respective wave lengths. The two interference filters with maximum transmittance at 570 m μ have a half-band width of 7 m μ . The filter transmitting at 440 m μ must have a half-band width of 16 m μ in order to pass sufficient light to elicit 10 μ a. from the photocell. The interference filters alone transmit extraneous light, usually at the higher wave lengths, and if Beer's law is to apply over a satisfactory range of color intensity, this extraneous light must be filtered out.

The combination of two Corning blocking filters, Nos. 4-97 and 3-69, gave satisfactory correction for the 570 m μ filters and with the 440 m μ filter, Corning filter No. 5-57 was used. The transmittance curve of each filter combination used in the present instrument was determined with a Cary recording spectrophotometer. The interference filters and Corning 4-97 blocking filter ordered to the above specifications were obtained in 1-inch squares from Baird-Atomic, Inc., Cambridge 38, Mass. The Corning 3-69 and 5-57 blocking filters are furnished with the respective interference filters by the manufacturer. A thin layer of mineral oil is placed between the interfaces of the filters, and the combination is bound together with black plastic tape around the perimeter. The composite filters are mounted with the diagonal of the square parallel with the direction of the slit in front of the cell. The side bearing the interference filter is placed next to the metal block.

The use of photovoltaic cells, *O*, which require no auxiliary power supply, contributes to the dependability of the photometer. The hermetically sealed cells have given very stable performance. If there is any significant difference in the current response of the three cells to light at 440 m μ , the one giving the largest response is used in the central photometer unit (cell 2).

The glass absorption cell, *P*, shown in detail in Figure 6, is a continuous tube which traverses all three photometer units.

The top part (for cells 1 and 2) is a 23-cm. section of 2.2-mm. (inside diameter) tubing, to which is sealed a 12/1 socket joint to receive the adapter leading from the Teflon coil. The bottom section (for cell 3) is a tube of slightly larger diameter, with a glass rod centered in it to reduce the effective depth of the cell to about 0.7 mm. A 6-cm. length of 3.0-mm. (inside diameter) tubing is first sealed to the top section. The rod (pulled out to 2.3 mm. in diameter and tapered at the upper end) is prepared for attachment by sealing it to a short section of 2.5-mm. (outside diameter) tubing. Two small holes are blown in the tubing a few millimeters below the end of the rod, and the tubing is cut off just below the hole. This combination is sealed to a 10-cm. section of 4.7-mm. (outside diameter) tubing carrying a $\frac{1}{2}$ 5/12 joint.

The final step is the insertion of the rod into the upper section and the completion of the seal without constriction of the holes. In order to keep the rod centered, it is wrapped at the mid-point with a 1-cm. wide ribbon of aluminum foil to give a close fit in the tube. The aluminum is subsequently dissolved out with 6*N* hydrochloric acid.

Alternatively, a 10-cm. length of 3-mm. (inside diameter) tubing can be sealed to the top section. A plain rod can be centered in position with the aid of two wrappings of aluminum foil and then fixed in position by spot welding the bottom of the rod to the outer tubing at two points opposite each other.

Each cell is calibrated individually in the assembled instrument. Although the diameters do not have to be precisely those given, the inside diameter of the upper section should be 2.1 to 2.3 mm. and none of the tubing should have more than a 1-mm. wall thickness. The refraction of light by heavier walled tubing will cause stray light to reach the photocell.

Three adapters for microphotometer tubes (*AD*, Figure 5) hold the absorption cells properly in the light beams and provide a 1.8 \times 11 mm. slit in front of the cell. It is necessary to cut off the cone-shaped end of each adapter and to drill an $\frac{11}{32}$ -inch hole through the bottom, to permit the absorption cell to pass completely through each holder. The side of the drilled hole just below the slit has to be filed out enough to allow the cell to fit flush against the slit. The cell and the top of the cabinet should be carefully marked, so that the cell can always be aligned in the block at the same height and with exactly the same side facing the light beam.

The cell adapters fit into the three aluminum housing blocks, *Q*, which are held together by the straps, *AE*, that fit into grooves on each side. The holes for the adapters are slotted and recessed to accommodate the keys and flanges on the adapters. Rectangular openings, $\frac{3}{4} \times \frac{3}{8}$ inch, are centered over the slits of the adapters. The

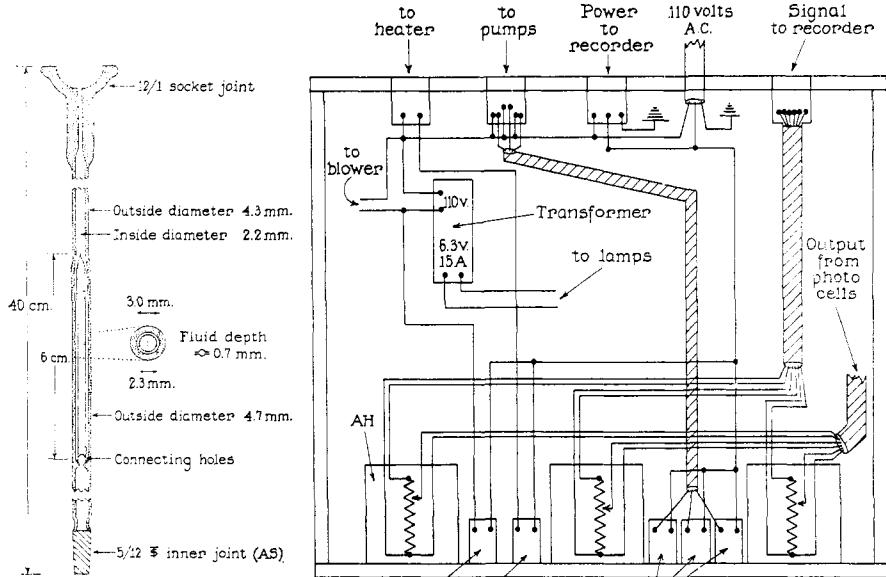


Figure 6. Absorption cell of photometer

Figure 7. Wiring diagram for photometer (Figure 5)

back of the block is cut (Figure 5) so that all light passing through the absorption cell can reach the photocell. The opening facing the back of the adapter is rectangular ($\frac{3}{8} \times 1\frac{1}{2}$ inches). The fit at the flange of the photocell must be light-tight.

To vary the current reaching the recorder from each photometer unit, a 1000-ohm variable resistance (Heli-pot AH , Figure 5) is placed as a shunt across the output from each photocell, as indicated in Figure 7. The turns-counting dial, AI , can be read to 1 ohm, and the current from each unit can thus be set precisely to give a reading of zero absorbance (10.00 μ a.) on the chart of the recorder when a blank solution is being analyzed. The potentiometers should not have a higher resistance than 1000 ohms; otherwise, the variation in the external resistance against which the photocell is operating will prevent identical performance at different settings of the dial.

Attainment of a steady base line depends in part on maintenance of constant voltage at the light source. The power for the three exciter lamps (15 amperes at 6.3 volts) was supplied in the initial instruments through a magnetic voltage regulator (Sola No. 20-04-095). A Sorensen electronic regulator No. 150 S with step-down transformer No. ST-7A (Sorensen and Co., South Norwalk, Conn.) is now used. This source of power has given an even more stable base line than that shown in Figure 2. The best results are obtained when the thermostatically controlled 50° water bath is on an alternating current line different from that supplying the photometer.

The heat generated by the exciter lamps is dissipated by forced air ventilation. A cage-type blower of 50 cu. feet per minute capacity (No. L-3198, Redmond Distributors, Inc., Owosso, Mich.) is mounted on the

rear wall of the photometer (Figure 5). Air is drawn up through a series of ventilation holes, AM , cut through the floor of the compartment directly beneath the lamps, K , and passes over the top of the partition, AK , to the blower. The outlet on the top of the cabinet is covered by a coarse metal screen ($1/4$ -inch squares). A few holes underneath the transformer allow a limited amount of air to be drawn up through the rear compartment. The same switch, AN , activates the blower and the transformer for the lamps (Figure 7). The entering air passes through a $1/2$ -inch thick glass fiber air filter cut to fit the base of the housing (Figure 5). In the latest instrument, this filter is placed below tube AQ , so that when the door is open, the filter can be slid out for occasional cleaning. Cellulose tissue should be packed loosely around cell P , where it emerges from the top of the cabinet.

In the latest model of the instrument, the running time indicator (Figure 1) is included in an enlarged photometer housing, and an additional on-off toggle switch for the timer is added to the panel. The resettable time indicator is a Cramer No. 640 E, total registration 9999.9 minutes, 115-volts, 60 cycles (R. W. Cramer Co., Inc., Centerbrook, Conn.).

Flowmeter. The plastic tube carrying the liquid flowing from the absorption cell passes through a horizontal section of 2-mm. bore glass tubing (Figure 1). A stopcock, AT , permits introduction of a bubble of air (a few millimeters long) either from an air line at 2 pounds' pressure or from a rubber atomizer bulb attached to AT . While this air bubble is being swept along at the same rate as the solution, measurement is made of the time required for it to travel between two permanent marks on the glass about 20 cm. apart. For the timing, a stopwatch is used which can be read to 0.1 second. The flow-

meter is initially calibrated by measuring accurately the volume of liquid delivered per 20 minutes at the effluent end (disconnected at the entrance to drain tube AU) for several different times of travel of the bubble between the two marks. A chart is prepared listing the calculated flow rates near 30 and 45 ml. per hour, in increments of 0.1 ml. per hour, with the corresponding times in seconds and tenths of seconds required for the air bubble to traverse the flowmeter. When size of tube mentioned above is used, a time of about 45 seconds is equivalent to a flow rate of 45.0 ml. per hour.

The fluid leaving the flowmeter enters an upright drain tube, AU , introduced to provide a constant pressure on the solution in the Teflon coil, and thereby to reduce the tendency for the formation of bubbles when the solution is at 100°. The drain tube is a 135-cm. length of 5-mm. diameter tubing inside a 140-cm. length of 10-mm. tubing. The solution spills over from the top of the inner tube and is led to waste from the bottom of the outer tube.

Recorder. The recorder is No. 69880-M2-M5-578 Special Speedomax Type G three-point current recorder (Leeds & Northrup Co., Philadelphia 44, Pa.) ordered with a range of 0 to 10 μ a., with gears to give a chart speed of 3 inches per hour (nominal), and with the M5 marking pen offset $\frac{3}{8}$ inch at the right end of the scale. With this recorder the signals from the three photometer units are printed consecutively at 4-second intervals as colored dots. The dots are closely spaced, and the chromatographic record consists of three curves (red, green, and yellow), each representing one of the photometer units. One of the inking pads of each color is replaced with a pad containing black ink, so that every fourth dot on each curve is black, which permits the simple method of integration of the curves referred to below. The chart paper [No. 578, Leeds & Northrup, or preferably, No. L 578S (green), Technical Charts, Inc., 189 Van Rensselaer St., Buffalo 10, N. Y.] onto which the recordings are plotted carries a vertical log scale marked from 0 to infinity, which reads directly as absorbance. The markings lengthwise along the chart are spaced 5 lines per inch, so that with the rate of elution of 30 ml. per hour and a chart speed of 3 inches per hour, each line on the chart is equivalent to 2 ml. of column eluent.

The recorder is designed to standardize itself every 48 minutes. This characteristic is a disadvantage when the peaks are to be integrated by the method described in the section on calculations. Therefore, it is desirable to disengage gear 92 (see manual of directions for Speedomax Type G, Model S recorders) and to standardize the recorder manually only at the beginning of each chromatogram.

The drain on the dry cell battery is so

small that many months of drift-free operation are obtained in this manner. As soon as there is a detectable change in the base line when the manual standardization is repeated at the end of an overnight run, the battery should be replaced.

The steadiness of the base line is dependent upon good condition of the contacts on the slide-wire and in the selector switch, both of which should be cleaned once a month.

Temperature Control. The jackets on the chromatograph tubes are kept at constant temperature by the circulation of water from an Aminco No. 4-96 bath (American Instrument Co., Silver Spring, Md.). To ensure an adequate flow, T-tubes ($\frac{5}{8}$ -inch outside diameter at the large end) are attached to the inlet and outlet lines at the bath and separate $\frac{1}{4}$ -inch (inside diameter) Tygon lines go from this T to the jacket of each 150-cm. column. Each short column is in series with one of the long ones.

Filtration is an effective way of controlling the growth of algae in the jackets and bath. To do this without restricting the flow, glass wool is packed loosely in the lower half cut from an 8-ounce polyethylene bottle, the bottom of which has been perforated by numerous $\frac{3}{16}$ -inch holes. A piece of stainless steel screen rests on the surface of the glass wool. This filter is fixed in position just underneath the return line in the cover of the bath and the top rim of the polyethylene cup is notched so that the overflow, if any, is directed toward the center of the bath.

For routine amino acid analyses of protein hydrolysates, the bath is operated continuously at 50° . When physiological fluids are to be studied, it is convenient to employ a second bimetal thermoregulator set at 30° and mounted in a cut-off section of the removable cover. A temperature change can be effected automatically by the same Gra-Lab timer that activates the solenoid-operated stopcock. For this purpose, a small relay (Type 41 ROZ-12000 ACS-Sil, Sigma Instruments Inc., Boston 85, Mass.) is wired in (Figure 8), so that when the relay is receiving 110 volts the 30° regulator is effective, and when the current is withdrawn from the relay, the 50° regulator is connected to the bath controls. The 110-volt line from the timer comes from the black outlet which is normally on, and is turned off when the clock reaches zero time. A three-position single-pole switch (SPDT, on-off-on) which can be mounted on the panel of the timer, provides for three modes of operation: continuous at 30° ; continuous at 50° ; and 30° while clock is running, 50° when it turns off.

RESIN AND REAGENTS

Resin. The preparation of columns of Amberlite IR-120 has been described (5). A resin preparation (fraction C)

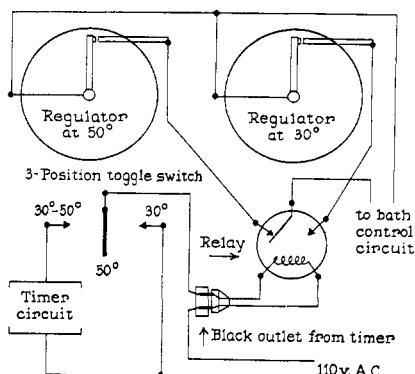


Figure 8. Wiring arrangement for use of two thermoregulators with constant temperature bath

containing particles about 40 microns in diameter is used to prepare the two 150-cm. columns and can be used for the 15-cm. column; the 15-cm. column is preferably prepared with particles 25 to 30 microns in diameter (fraction B), and the 50-cm. column is always made from this finer fraction. Before a freshly poured 150-cm. column is operated at 50° , deaerated buffer at pH 3.25 is pumped through for 3 or 4 hours while the column is at room temperature. When the temperature is raised to 50° , the pressure required to pump buffer through the column at 30 ml. per hour should be 30 to 40 pounds. During the first few analyses, the pressure may increase slightly, but should level off below 50 pounds. If the columns are used at 30° , which is necessary for the analysis of physiological fluids, the pressure will be 12 to 14 pounds higher than that observed at 50° .

A column that requires a pressure greater than 60 to 70 pounds to maintain a flow rate of 30 ml. per hour is approaching the maximum pressure at which the present equipment can be operated without leakage. In such circumstances, the resin should be withdrawn from the tube, a small percentage of the finest particles should be removed by hydraulic separation, and the column should be repoured. Because a very small amount of colloidal material in the suspension of resin can cause the resistance to be too high, it is always desirable to pour the column initially from particles that have been freshly fractionated by the hydraulic method or settled several times from buffer. If a properly packed column gives less resolving power than is indicated in Figures 2 and 9, a fraction of resin of smaller average particle size should be prepared.

Buffers. The compositions of the buffers used as eluents in the chromatographic analysis shown in Figure 2 are given in (5).

The buffer reservoirs (Figure 1) are filled by withdrawing 2 to 4 liters of buffer from the main supply, which is

stored at 4° , warming it to room temperature before adding the specified quantities of detergent (BRIJ 35) and antioxidant (thiodiglycol), and filtering the solution into the corresponding reservoir through a fluted filter.

The smaller amount of buffer at pH 3.25 stored under oil (top of Figure 1) is used to re-equilibrate the columns after the sodium hydroxide wash. The dissolved air in this portion of buffer is removed by boiling 2 liters of filtered buffer (containing BRIJ 35 and thiodiglycol) in a 4-liter Erlenmeyer flask. A boiling stick is placed in the flask to prevent bumping. The buffer is boiled for 10 minutes and is then immediately introduced under the oil in the reservoir bottle through an open funnel carrying a 12/5 ball joint which fits onto the siphon tube of the reservoir bottle.

The 0.2N sodium hydroxide used to wash the columns contains BRIJ 35 but no phenol or thiodiglycol.

Ninhydrin Reagent. The composition of the reagent solution designed specifically for use with the recorder combines features of both of the ninhydrin reagents described previously (6, 9).

Stannous chloride, instead of hydrindantin, is used as the reducing agent (6) in a 75% methyl Cellosolve-25% 4M sodium acetate buffer (9). This reagent is stable for at least a month, and contains a concentration of reduced ninhydrin below that which normally causes precipitation of hydrindantin in the lines. Methyl Cellosolve, peroxide-free grade, which should give a colorless solution when tested with potassium iodide (6, 9), is purchased directly from the manufacturer (Union Carbide Chemicals Co., 30 East 42nd St., New York 10, N. Y.) in 5- or 55-gallon drums. The fact that the solvent is packaged under nitrogen in light-proof containers helps to keep it peroxide-free. The 4M sodium acetate buffer at pH 5.5 (9) is usually prepared in 10-liter quantities and can be stored at room temperature without a preservative. Both the methyl Cellosolve and the buffer are filtered before use.

The reagent solution must be prepared and introduced into the reservoir in the absence of oxygen. Three liters of methyl Cellosolve and 1 liter of 4M sodium acetate buffer are stirred magnetically in a 4-liter aspirator bottle, the bottom outlet of which is provided with a 2-foot length of Tygon tubing ($\frac{1}{4}$ -inch inside diameter, $\frac{1}{16}$ -inch wall thickness) that carries a $\frac{1}{4}$ 14/35 inner ground joint. During this time, a slow stream of nitrogen (purified grade, The Matheson Co., East Rutherford, N. J.) is bubbled in through the tube, with the joint held at the level of the top of the bottle. After the mixture has been stirred for 15 minutes (to displace air by nitrogen), 80 grams of ninhydrin (Pierce Chemical Co., P.O. Box 117, Rockford, Ill.) are dissolved, followed by 1,600 grams of stannous chloride dihydrate (analytical reagent grade, Mallinckrodt). While the stannous chloride is going into solution, the

Tygon tube is clamped off near the joint, and the nitrogen line is transferred to an inlet in a two-hole rubber stopper in the top of the bottle. After solution is completed, the filling bottle is moved to a position above the ninhydrin reservoir, and the two are connected by means of the Tygon tube and the 14/35 joint. Before the stopcock is opened, all of the gas in the connecting line is allowed to bubble out through the filling bottle. The stopcock to the reservoir is then opened. Slight pressure is applied by intermittently placing a finger over the outlet of the nitrogen line in order to complete addition of the ninhydrin solution in about 5 minutes.

When the instrument is first set up, nitrogen is run in through the stopcock and out through the third bottle (Figure 1) for 60 minutes to displace air in the system. By using 2½-gallon bottles for the oil, the level of oil in the air-oil bottle can be maintained at all times higher than that in the nitrogen-oil bottle, thus ensuring a slight positive pressure of nitrogen. In the event of a leaking connection, therefore, air cannot enter against the pressure of escaping nitrogen. If the system is leak-free, the oil level should return to the same mark each time the ninhydrin reservoir is filled.

Standard Mixture of Amino Acids.

The instrument was standardized by the use of a synthetic mixture of amino acids, each component of which had been found by the fraction collector method (5) to be quantitatively recoverable in a single peak from an ion exchange column.

To facilitate the weighings, the solution was prepared on a relatively large scale by transferring $1250 \pm 2 \mu\text{moles}$ of each amino acid (half of this quantity of cystine) into a 500-ml. volumetric flask. The flask was half filled with water and 5 ml. of concentrated hydrochloric acid were added to dissolve the less soluble amino acids. The final solution, containing 250 μmoles of amino acid per ml., was transferred in 5.2-ml. quantities to 10-ml. glass ampoules which were sealed under nitrogen and stored in the deep freeze. A sample to be analyzed was prepared by diluting 1 ml. of the standard solution with 4 ml. of pH 2.2 buffer (8) containing BRIJ 35 and TG, thus providing 2-ml. aliquots for analysis which contain 1.00 μmole of each amino acid (cystine considered as half cystine).

OPERATION OF EQUIPMENT

The complete amino acid analysis of a protein hydrolyzate, for which the following procedure is applicable, requires the use of one of the 150-cm. columns (1A or 1B in Figure 1) to determine the neutral and acidic amino acids, and the short column (column 2, Figure 1) to determine the basic amino acids. Duplicate samples are thus needed, one for each column, and it is convenient to add aliquots from the solu-

tion to be analyzed to both columns at the same time in the morning. When purified proteins are analyzed (3), the hydrolyzate from about 5 mg. of protein may be brought to a final volume of 5 ml. at pH 2. Aliquots (2 ml.) of the solution are used for each column. As a general precaution, any solution for analysis is centrifuged or filtered free from insoluble material before being applied to the resin.

The short column is run first, requiring about 5 hours, and the effluent from the long column is then analyzed during an overnight operation. As only the acidic and neutral amino acids are eluted from the long column during the analysis, a wash with sodium hydroxide is necessary to remove the remaining basic constituents before the column is used over again. The regeneration can be completed conveniently in 24 hours, so that each long column may be used on alternate days; while one is being employed for an analysis, the other is being regenerated. The short column seldom needs regeneration between uses.

When operated according to the following procedure, the instrument requires only part-time attention during the working day. The description refers to Figure 1, unless otherwise noted. Two interval timers (X-Ray Division, General Electric Co., No. E 1208 C), numbered 1 and 2, are used to time various steps in the operation of the respective columns.

I. Conclusion of Previous Day's Analysis. The analysis of a sample added to the 150-cm. column—e.g., 1B—the day before is completed through phenylalanine by 9 A.M. (about 1000 minutes on the running time indicator, equivalent to 500 ml.).

1. Turn the Gra-Lab timer off. This de-energizes the solenoid-operated valve to connect the pump with the pH 3.25 buffer reservoir. (This step is required to pump buffer at pH 3.25 through the line for at least 30 minutes before Step II-6).

2. Measure and record on the data sheet the combined flow rate of pumps 1 and 3. If the three readings that agree to 0.1 second are within 0.2 second of the desired value for 45 ml. per hour, the variation is normal. If the rate is off by 0.4 second, the chromatogram is satisfactory, but the system should subsequently be examined for leaks and the performance of the pumps should be checked.

3. Turn the stopcock on the ninhydrin line to drain and stop pump 3.

4. Manually standardize the recorder. (When there is a detectable change in the base line as a result of restandardization after overnight operation, the battery needs to be renewed.)

5. Switch off the recorder, remove the recorded curve, and attach the chart paper for the next run.

II. Start of New Analysis.

Samples are applied to both columns shortly after 9 A.M. In preparing to add the sample to the 15-cm. column, several precautions are necessary to prevent contamination by ammonia, the presence of which can give rise to a broad zone underlying the regular ammonia peak on the effluent curve.

1. Remove the ball joint and the silicone washer from the top of column 2 and wipe the ball and the socket with a moist cloth or tissue, to remove any ammonium salts that may have deposited from the atmosphere. Place the joint (usually bent at a 60° angle) over the edge of a 30-ml. beaker, just rinsed with distilled water. Turn on pump 2 for a few minutes to add about 1 ml. of buffer to the beaker. Withdraw the buffer above the resin surface with the aid of a wiped pipet and add to the beaker.

2. Add the sample of the amino acid solution to be analyzed (at about pH 2) with a calibrated, bent-tip pipet to the surface of the resin that is just clear of free liquid. The volume of sample applied is usually 2 ml. Air pressure can be used to drive the sample in, if the rate does not exceed 30 ml. per hour.

3. Prepare column 1A, which has been equilibrating with pH 3.25 buffer overnight, for the sample by pipetting out or aspirating off the buffer above the resin. When a 2-ml. sample (at pH 2) is applied, an air pressure of 5 pounds (25 cm. of mercury) can be used to drive it in. Set interval timer 1 for the time—e.g., 15 or 20 minutes—which experience has shown is required to drive the sample almost completely into the resin.

4. While the sample is passing into the long column, complete the addition to the short column in 3 to 4 minutes by applying air pressure from a wiped ball joint attached to an air line connected by a tee to the line shown at the top of Figure 1. Wash the sample in with three 0.2-ml. aliquots of the pH 5.28 buffer taken from the beaker. The use of a bent-tip micropipet (6) makes it easier to rinse down the walls of the tube thoroughly. Replace the buffer above the resin, using the same pipet (bent-tipped) with which it was withdrawn. Replace the silicone washer with the aid of forceps, taking care to center it, and attach the buffer line from pump 2. A small air bubble just below the ball joint can usually be avoided, but is not harmful.

5. Before the pump to the 15-cm. column is started, check the rate of flow of buffer alone from pump 1, which has been pumping buffer through the column and the coil for at least 30 minutes (since Step I-3), and should show a rate of 30 ± 0.2 ml. per hour. After this measurement of rate, turn the stopcock of column 1B from recorder to drain, and switch off pump 1. Start pump 2, and turn the stopcock of column 2 to the recorder position. Set interval timer 2 for 20 minutes, the time required for the forerun from the short column.

6. During the forerun on column 2, the addition of the sample to column 1A can be completed. Wash in the sample with three 0.2-ml. aliquots of pH 3.25 buffer. Fill the space above the resin with pH 3.25 buffer, taking care not to disturb the surface of the resin during the addition. Transfer the buffer line leading from pump 1 from the used column (1B) to column 1A, using two clamps for the connection. Switch on pump 1, and set timer 1 for 20 minutes to time the forerun, which in this case takes place with the effluent directed to drain. When timer 1 rings, turn off pump 1 until the chromatogram is started in the afternoon (Step V).

7. While the forerun on column 2 is taking place, there is also usually time to apply the 0.2N sodium hydroxide wash to the used 150-cm. column (1B). Pipet out or aspirate off the buffer to within 2 cm. of the surface of the resin. Fill the tube with sodium hydroxide and attach the ball joint from the reservoir. Open the stopcock or clamp and allow the alkali to enter the column under 5-pounds pressure during the day.

III. Recording from Column 2. When timer 2 rings after 20 minutes have elapsed, switch on the running time meter (preset to zero), switch on pump 3 (ninhydrin), and turn the ninhydrin stopcock from the drain to the recorder position. Set timer 2 for 39 minutes as a reminder that at 40 minutes on the running time indicator the recorder should be switched on. Adjust the chart paper so that the instrument prints on one of the heavy horizontal lines. The effluent volume is 20 ml. at this time, and the chart can be so marked on the margin. Manually standardize the recorder after it has been on for at least 2 minutes. Adjust the base line of each of the three cells to zero by means of the corresponding Heliopot and observe for the first few minutes, to make sure that the curve has leveled off. Measure the combined flow rate of pumps 2 and 3.

To record the tyrosine-phenylalanine peak from the 15-cm. column, turn on the recorder at 16 minutes instead of 40 minutes, and set the Heliopots at the readings used the day before. Make final adjustment of the base line after the curve has leveled off about 25 minutes later. The peak from chlorotyrosine, if present, emerges just after tyrosine-phenylalanine, and may delay the final adjustment a few minutes.

The above operations are completed by about 10:30 A.M.; the following operations remain to be completed in the latter part of the afternoon.

IV. Completion of Analysis on 15-Cm. Column. The chromatogram is finished after 280 minutes on the running time indicator (140 ml.). Check the combined flow rate. (If the pumps are not functioning properly, the flow rate of pump 2 can be checked independently at this time by turning the ninhydrin stopcock to drain and checking the rate of pump 2 30 minutes later.) Turn off the re-

corder and manually advance the chart about 2 inches. Turn off and reset the running time indicator.

V. Recording from Column 1. When the short column is finished (about 3 P.M.), pump 1 can be switched on and the stopcock from column 1A turned to the recorder position at the same time that the stopcock on column 2 is turned to drain. The ninhydrin stopcock is normally left in the recorder position without interruption. Set the timer which energizes the solenoid-operated valve for the desired number of hours and minutes and turn on the timer switch and that of the running time indicator. (In order to produce the change in pH of the effluent just after cystine in Figure 2, the valve was activated 8 hours and 20 minutes after the start of the analysis.) Set timer 1 for 39 minutes as a reminder that at 40 minutes on the running time meter the recorder should be turned on and adjusted to print on a heavy horizontal line. Standardize the recorder and adjust the three photometers to zero. Check the combined flow rate of pumps 1 and 3.

VI. Completion of Regeneration of Other 150-Cm. Column. Before leaving in the afternoon, remove the sodium hydroxide line from the column. The alkali should have traversed about two thirds the length of the column, as evidenced by a visible change in the color of the resin. Withdraw the alkali to within 2 cm. of the resin surface and replace with buffer at pH 3.25. Attach the line from the 2-liter reservoir that contains pH 3.25 buffer under oil. The column can thus be equilibrated with the buffer by the following morning, and be ready for the next sample.

COMMENTS ON PROCEDURE

Matching of Columns. It is advantageous to have two 150-cm. columns, closely matched as to performance, so that the time setting for the solenoid-operated stopcock can be the same for each. It is also helpful in comparing chromatograms if the peak from a given amino acid occupies the same position on the two effluent curves. Slight variations in the inside diameters of the chromatograph tubes can cause differences in retardation volumes, but this can be corrected for by adding to or subtracting from one of the columns a few centimeters of resin until the two effluent curves are essentially identical.

After columns have been in use for several months, there may be a decrease in the height of the resin bed. Appropriate quantities of fresh resin can be added to both columns to restore the original length. If insoluble material accumulates on the surface of a column and is not removed by the sodium hydroxide wash, the top layer of the bed should be withdrawn and replaced with clean resin.

Foreruns. The 20-minute forerun, which is not registered on the running time indicator, corresponds to the approximate time required for a portion of the effluent to be pumped from the sintered plate of the column through the heating coil and associated tubing and reach the photometer. In the case of the 150-cm. column, this forerun moves the sample part-way into the resin bed, so that amino acids do not diffuse back into the buffer above the resin surface while the long column is being allowed to stand, with the pump off, during the day.

With the 15-cm. column, the forerun is purposely made before the ninhydrin reagent is pumped into the effluent. The effluent which emerges during these 20 minutes carries through nearly all of the relatively unretarded acidic and neutral amino acids. If the ninhydrin is added from the start, the initial concentration of the blue colored compound formed in the reaction (6) is so high that crystals of the compound may form in the heating coil and absorption cell.

The 40-minute periods after the ninhydrin is turned into the line and before the recorder is turned on allow 20 ml. of effluent admixed with ninhydrin reagent to flow through the photometer. By this time equilibrium conditions will usually have been reached, thus permitting a stable setting of the base line and an accurate check of the combined rate of flow from the pumps.

Temperature of Photometer. A change of as much as 5° C. in room temperature will cause a measurable but not serious drift in the base line. The photometer is preferably mounted off the bench top and not in direct line with currents of cool air.

Continuity of Operation. The photometer, the 50° bath, and the boiling water bath are normally left on continuously, including weekends. About 2 or 3 hours are required for the photometer to come to equilibrium after being turned on. If the deaerators have been allowed to cool down, it may be necessary to introduce a little air through the joint of the reservoir to reactivate the surface of the glass wool. Whenever the water jackets on the chromatograph tubes are allowed to cool to 30° or below, buffer should be free to enter the top of the column to prevent air from being sucked back through the sintered plate as the buffer in the column contracts. Alternatively, an S-tube of 2-ml. capacity can be placed on each drain line of the stopcock manifold. To minimize deterioration of the sintered plates, columns should not be left in contact with 0.2N sodium hydroxide longer than necessary.

At the close of an analysis, or whenever the apparatus is to be allowed to stand without fluids being pumped through the coil, the Teflon coil should

always be pumped free of ninhydrin reagent by passing one of the buffers through the coil for 30 minutes. If ninhydrin reagent (rather than buffer) is allowed to stand in the boiling water bath, the prolonged heating causes the formation of insoluble products which may clog the coil and necessitate its replacement. If, in the event of precipitation in the coil, undiluted ninhydrin solution can still be pumped through, it is usually possible thus to remove the insoluble material. A film that sometimes has been observed to deposit in the absorption cell can also readily be dissolved out in this manner. A brown deposit that formed in the first few loops of the heating coil after a year of use was readily removed with benzene. Bubbles in the absorption cell may be washed out by allowing solution to flow slowly back up through the cell from the drain tube, *AU*.

Performance Data. It is useful to keep daily records of the pressures registered on each gage and the settings of each pump and of each Heli-pot. Failure of a gage to reach or maintain the expected pressure is a ready indication of some fault in the system.

CALCULATIONS

Integration by Height-Times-Width Method. The peaks on the effluent curves (Figures 2 and 9) are usually integrated by multiplying the height of the peak by the width at half the height. This method of integration is rapid and satisfactorily accurate. The height of the peak in absorbance is easily determined from the recorder chart. In the beginning of this work, the width of each peak on the chart was estimated to 0.1 mm. with a ruler graduated to 0.5 mm. As the Speedomax multipoint recorder prints a dot every 12 seconds on each curve, the number of dots on the curve above the half-height line can provide an accurate measure of the width of the peak in terms of time (seconds). To facilitate the counting of the dots, every fourth dot is printed in black. The width of a peak can be measured to within one tenth of the distance between dots, and hence the width of a peak 40 dots wide can be obtained accurately to 0.25%.

To integrate a peak, the procedure is as follows.

Records are kept on multilithed data sheets ($8\frac{1}{2} \times 11$ inches) that have the following column headings: (1) amino acid; (2) base line in absorbance units; (3) height in absorbance units; (4) half-height on chart; (5) net height, H ; (6) W (width) in dots, seconds/12; (7) $H \times W$; and (8) micromoles $H \times W/C$, where C is a constant for the given amino acid. Several blank columns are included on the sheet for further calculations. First, the base line is read to 0.001 absorbance unit. If the curve

permits a reading of the actual base line at both sides of the peak, the average of the two values is used. For valine, which emerges just after the breakthrough of the pH 4.25 buffer, the base line following the peak should be taken. The height of the peak on the chart is then read from the center of the top dot or dots, and noted in column 3. The net height (H , column 5) is ob-

tained by correcting the value in column 3 for the base line. The half-height on the chart (column 4) is determined by taking half of the value in column 5, and adding or subtracting the baseline correction. Fine lines are drawn to mark the half-height on the ascending and descending sides of the peak.

In order to use every fourth black dot as an aid in counting the total number of dots (column 6), the first black dot above the marked line on the ascending side of the curve is skipped and all subsequent black dots around to the mark on the descending side are counted, jotted down, and multiplied by 4. The width measurement (W , column 6) is completed by adding to this figure the distances between the penciled lines and the first black dots on the right and the left sides of the peak; to determine these distances, the space between each two individual dots is counted as 1, and the fractional distance from each penciled line to the dot next above it is estimated to 0.1 unit. To facilitate counting of the black dots in dense sections of the curve, use may be made of the fact that each square on the chart paper equals five black dots.

For the final calculations, the net height, H (column 5), of the peak is multiplied by W (column 6). The constant by which $H \times W$ is divided to give micromoles of a given amino acid is determined by calibrating the apparatus with a synthetic mixture of amino acids. In most cases, the value of this constant varies only slightly from one amino acid to another; the constant ($H \times W$ per μ mole) is a function of the color yield of the given amino acid in the ninhydrin reaction and the dimensions of the absorption cell. As the data show, once established, this constant is valid for a given instrument under a wide variety of conditions.

If the height of the peak on the recorder chart exceeds an absorbance of 1.4, the same method of integration is applied to the lower curve obtained using the thinner absorption cell (cf. Figure 6). The factor by which the $H \times W$ from the lower curve should be multiplied to give values comparable to those from the upper curve is obtained by averaging the ratio of the two integrations for several relatively high peaks for which the upper curve can be integrated accurately (height < 1.4). The factor for the cell in the present instrument is 2.58.

In the analysis of unknowns, $H \times W$ is divided by C to give the micromoles of each amino acid in the sample added to the column. The time required for an experienced operator to integrate a typical chromatogram—e.g., Figure 2—by the $H \times W$ method is about 1 hour.

In Table I are given the constants thus obtained for 50 compounds with the recorder used in this study. The list includes the compounds most commonly found in physiological fluids, and referred to in connection with Figure 9.

Table I. Observed Constants for Integration of Peaks Obtained from Amino Acids and Related Compounds

Values for C in $H \times W/C = \mu$ mole are the constants per μ mole of ninhydrin-positive compound determined with a given photometer. If the integration has been made by addition of absorbance readings, the applicable constant is 0.106 times C .

Compound	C
50° System (Figure 2)	
Cysteic acid	25.5
S-Carboxymethylcysteine	23.1
Methionine sulfoxides	25.0
Aspartic acid	25.2
Methionine sulfone	25.2
Threonine	25.4
Serine	26.1
Glutamic acid	26.3
Proline (440 m μ)	6.37
Glycine	25.6
Alanine	26.0
Half-cystine	14.4
Valine (eluted at pH 4.25)	27.2
Valine (eluted at pH 3.25)	25.1
Methionine	25.6
Isoleucine	27.3
Leucine	27.6
Tyrosine	27.0
Phenylalanine	26.7
Glucosamine	26.6
Galactosamine	Ca. 23.2
Tryptophan	22.2
Lysine	30.7
Histidine	28.0
Ammonia	26.9
Arginine	27.5
30°-50° System ^a (Figure 9)	
Phosphoserine	Ca. 27.2
Glycerophosphoethanolamine	20.6
Phosphoethanolamine	15.7
Taurine	25.1
Urea	0.97
Hydroxyproline (440 m μ)	2.54
Asparagine	23.4
Glutamine	Ca. 21.8
Sarcosine	6.48
Citrulline	26.5
α -Aminodipic acid	23.7
α -Amino- <i>n</i> -butyric acid	24.9
Cystathione (2 NH ₂ groups)	31.9
Half-homocystine	25.4
β -Alanine	11.4
β -Aminoisobutyric acid	12.5
Hydroxylsine	29.0
γ -Aminobutyric acid	27.3
Ornithine	29.9
Ethanolamine	20.5
Ammonia	24.7
Lysine	28.6
1-Methylhistidine	22.5
Histidine	26.5
3-Methylhistidine	Ca. 22.5
Anserine	18.2
Tryptophan	18.5
Creatinine	0.72
Carnosine	21.7
Arginine	25.4

^a Constants given for cysteic acid through galactosamine with 50° system also apply to 30°-50° system.

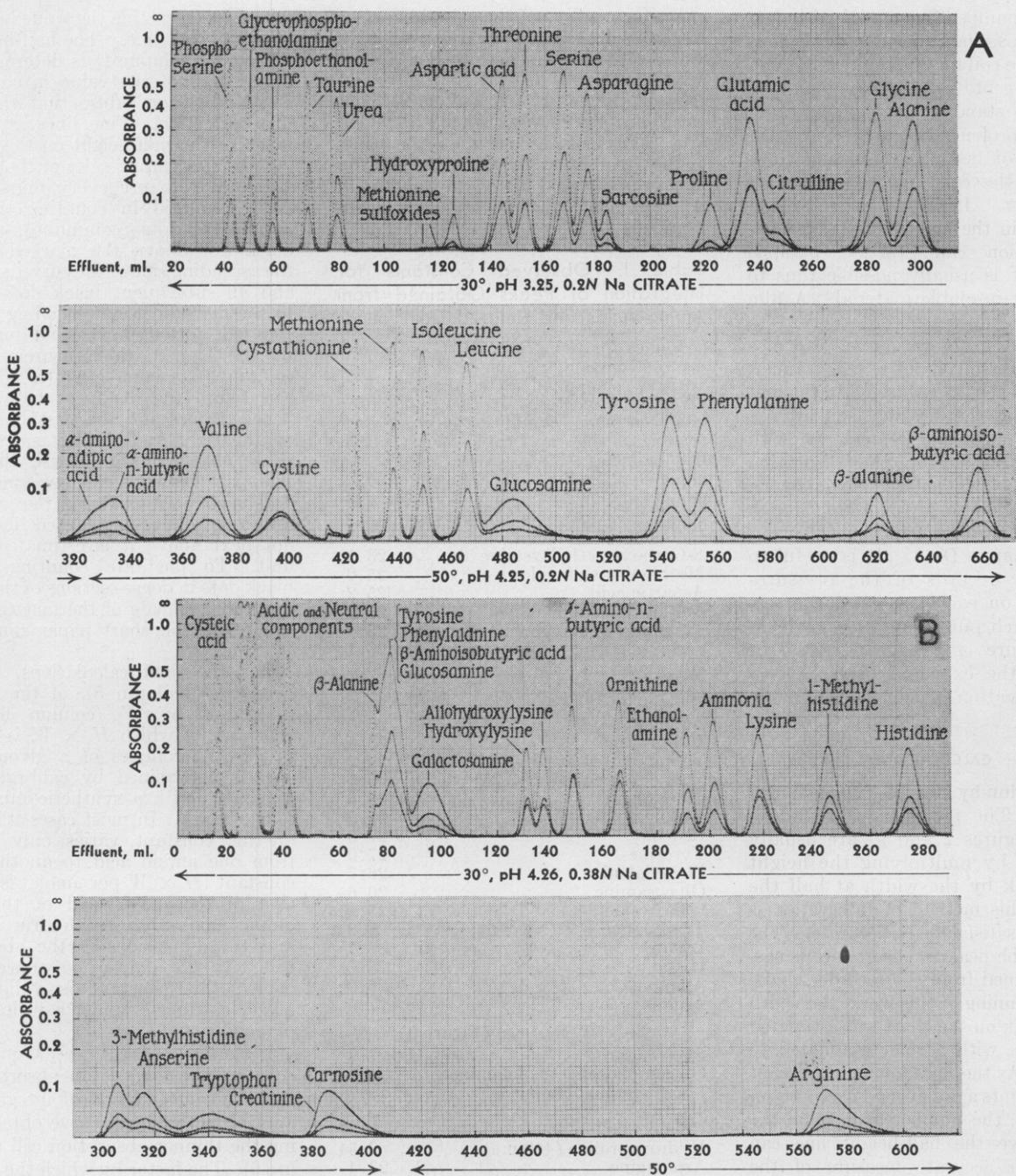


Figure 9. Chromatographic analysis of mixtures of amino acids and related compounds most commonly encountered in physiological fluids

- A. 150-cm. column of Amberlite IR-120 at 30° and 50°
- B. 50-cm. column at 30° and 50°

Glutamine has the same rate of travel as asparagine; cysteic acid and phosphoserine are not separated; galactosamine, if present, emerges with tyrosine and phenylalanine in A; homocystine is eluted as a zone preceding but partially overlapping β-alanine; felineine is slightly more retarded than citrulline, but overlaps it; behavior of glutathione is discussed in the accompanying paper (5). The change in the influent buffer was made at 325 ml., and the break-through of the pH 4.25 buffer is observed at 415 ml.

The absolute values of the constants depend primarily upon the dimensions of the absorption cell (Figure 6). If a given recorder has not been calibrated for all compounds under study, it is probable that additional constants can be calculated with fair accuracy from the ones already determined by the user, on the assumption that the relative differences between the constants given in Table I will be generally applicable.

When the fraction collector method is used, the ninhydrin reagent (9) gives

color yields that are relatively independent of the pH or ionic strength of the sample. With the lower concentration of reduced ninhydrin in the reagent designed for the recorder, however, the constants vary slightly with the nature of the eluting buffer. Thus, two constants are given for valine in Table I, and the one used depends upon whether valine is eluted at pH 3.25 or 4.25. Similarly, two series of constants are given for the basic amino acids which are eluted by different buffers in the 50°

and the 30°–50° systems illustrated in Figures 2 and 9.

Integration by Addition of Absorbance Readings. The total ninhydrin color in a given peak can, of course, be obtained by addition of the absorbance values read from the recorded curve, in a manner strictly analogous to that used for the determination of the amount of an amino acid in a peak when the fraction collector method is used. The addition takes more time than the measurement of H and W , but is pref-

Table II. Reproducibility of Results with Known Mixtures of Amino Acids

Data obtained from chromatograms similar to the one illustrated in Figure 2. Recoveries calculated using integration constants, C , determined previously at a load of 1 μmole of each component (Table I)

Compound	Quantity of Each Amino Acid Added to Column, μmoles								
	1 (Six Chromatograms)			4	2	0.5	0.25	$1/8$	
Av., %	Av. dev. from mean, %	Max. dev. from mean, %			Recoveries, %				
Cysteic acid	100	0.6	1	Off scale	99	99	100	100	95
Aspartic acid	100	1.0	2	101	99	99	100	101	99
Methionine sulfone	99	1.0	2	89	92	101	102	102	101
Threonine	100	0.8	2	101	98	96	101	102	94
Serine	99	0.3	2	102	99	104	100	101	101
Glutamic acid	100	0.7	2	101	98	100	100	100	94
Proline	100	1.6	3	99	99	101	100	99	100
Glycine	99	1.3	3	101	97	99	99	104	96
Alanine	99	1.0	2	98	98	100	98	104	97
Half-cystine	100	1.7	2	100	99	101	98	100	98
Valine	100	1.7	2	Off scale	98	101	100	103	101
Methionine ^a	100	1.5	3	98	100	100	100	100	94
Isoleucine	100	0.7	1	101	100	102	99	99	94
Leucine	100	0.3	1	101	98	101	100	100	95
Tyrosine	99	0.3	1	100	98	100	99	102	98
Phenylalanine	100	0.5	1	100	98	102	99	100	97
Lysine	100	0.3	1	Off scale	101	100	99	100	103
Histidine	100	0.2	1	98	101	100	99	100	101
Ammonia	100	1.0	2	86	97	102	101	98	103
Arginine	100	0.8	1	97	98	98	100	98	102

^a Calculated as methionine plus methionine sulfoxides.

erable for very small peaks such as the methionine sulfoxides (Figure 2), overlapping peaks such as 3-methylhistidine and anserine (Figure 9, B), or a very asymmetric peak. The determination of cystine in protein hydrolysates is a common instance requiring the addition of absorbance readings. The presence of *meso*-cystine, formed as a result of racemization during hydrolysis (2), leads to the appearance of a markedly asymmetric cystine peak that cannot be integrated by the $H \times W$ method.

When integration is performed by the addition procedure, the absorbance is read from the curve at 1-ml. intervals along the chart. As the distance between the vertical lines on the chart paper corresponds to 2 effluent ml., readings are taken on each vertical line and midway between each pair of lines. The values are added and a base-line correction is made by subtracting the average base-line reading multiplied by the number of values taken. The result is converted to micromoles of amino acid by dividing the net sum by 0.106 times the appropriate constant taken from Table I.

The experimental observation that a single factor can be used to convert any of the height times width constants to the constant applicable to the direct absorbance measurement is evidence for the fundamental validity of the $H \times W$ method. In a series of 24 comparisons of the two methods of integration, the factor of 0.106 was obtained with an average deviation from the mean of only 0.7%. Thus, even when peaks of various degrees of symmetry are integrated, the height times the width at half the height gives a very close estimate of the quantity of amino acid present. If this

were not the case, the $H \times W$ method would not give the precision noted below when different quantities of amino acids are determined, as the shapes of the peaks change detectably with increase in load.

Precision. The precision of the chromatography and of the $H \times W$ method of integration was checked by a series of analyses in which the quantity of each amino acid added to the column ranged between 4 and $1/16$ th μmole . The results, given in Table II, show that a satisfactory integration can be obtained when the size of the peaks varies 64-fold. At the lowest load, which corresponds to only 5 to 10 γ of each amino acid, the lower limit of the method is being reached. At the high load of 4 μmoles , two substances, methionine sulfone and ammonia, show low yields. The former might deplete the reduced ninhydrin in the reagent solution. In the case of ammonia, the yield of color is known to be particularly dependent upon the amount of hydridantin present, and hence a reduced color yield can be expected when the amount of ammonia in the sample approaches the molar concentration of stannous chloride in the reagent.

From Table II, it can be seen that the method is capable of giving reproducible recoveries of better than $100 \pm 3\%$ for quantities ranging from 0.25 to 2 μmoles of amino acid. The fact that the method can give useful results with amounts as low as $1/16$ th μmole is largely a result of the stability of the base line. Immediately before and after a peak the base line is usually constant to ± 0.001 absorbance unit, which is a much greater constancy than can be obtained normally when fractions are collected and

analyzed in individual photometer tubes (6). A peak with a height of only 0.02 absorbance unit can therefore be integrated with fair results. In work with simple peptides, for example, the molar ratios of the constituent amino acids can, if necessary, be determined on samples no larger than those required for paper chromatography.

ANALYSIS OF PHYSIOLOGICAL FLUIDS

An alternative mode of operation of columns of Amberlite IR-120 (5) permits the use of the recorder to be extended to the determination of the ninhydrin-positive compounds in blood plasma, urine, and extracts of mammalian tissues. The positions of the approximately 50 compounds with which the analyst is principally concerned in such studies are shown in the curves reproduced in Figure 9.

The analysis is begun with the 150-cm. column at 30° , and the Gra-Lab timer is set to accomplish both the change to an eluent at pH 4.25 and the increase in temperature of the circulating water bath to 50° at about 11 hours, in order to cause the change in the pH of the effluent to occur just after cystine has emerged from the column (Figure 9, A). The complete run on this column, through β -aminoisobutyric acid, requires about 22 hours. The basic amino acids and related compounds are determined by a second overnight run on a 50-cm. column operated at 30° and 50° with a buffer at pH 4.26, 0.38N in sodium (Figure 9, B). The 50-cm. column is usually used over again without a wash with alkali. If a wash with sodium hydroxide (0.38N) is made, more than 200 ml. of 0.38N pH 4.26 buffer needs to be pumped through before the column is used.

Only minor additional equipment is needed to make the recorder suitable for the analysis interchangeably of protein hydrolyzates (Figure 2) and more complex mixtures that require the system illustrated in Figure 9: an additional reservoir bottle for the pH 4.26 buffer, connected to a fourth deaerator, and a 2-mm.-bore three-way stopcock placed in the line to pump 2 to permit buffer to be drawn either from the pH 5.28 reservoir (when the 15-cm. column is being used) or from the reservoir containing the 0.38*N* buffer at pH 4.26 (for the 50-cm. column).

In shifting from one buffer to the other, at least 30 ml. should be pumped through the lines before the connection to the new column is made. If a given buffer line has not been used for a number of days, the buffer that has been standing in the deaerator may be discolored (owing to the phenol present) and should be pumped out before the column is started.

The 50-cm. column poured from resin fraction B [(5), Table I] should require an operating pressure of about 45 pounds, without added resistance in the line. A higher setting of pump 2 may be necessary to obtain 30 ml. per hour when the 50-cm. column is used, unless the operating pressures for it and the 15-cm. column are made nearly equal. Such an equalization may be accomplished by removing the added pressure regulator (*B*, Figure 1) from the line when the 50-cm. column is being used, or by modifying the design of the pressure regulator so that there is a bypassing arm with a section of Tygon tubing (1/16-inch inside diameter, 3/16-inch outside diameter) which can be clamped shut when the 15-cm. column is in operation.

When human blood plasma is analyzed by the procedure shown in Figure 9, 24 ninhydrin-positive constituents can be determined (17). A protein-free filtrate corresponding to 4 ml. of plasma constitutes an appropriate load for such an analysis. For the study of human urine (16), a 2-ml. sample of urine (specific gravity 1.02, filtered and brought to pH 2) is used for the 150-cm. column, and a 1-ml. sample for the 50-cm. column. The resulting chromatograms show the numerous peaks attributable to the constituents known to occur in urine, and, in addition, a number of small ninhydrin-positive peaks derived from compounds not yet identified. The analysis of tissue extracts is discussed in (5); the recording equipment has been used in the study by Tallan, Moore, and Stein (19) of the amino acids in human brain. Tallan has also found the 50-cm. column suitable for the determination of anserine and carnosine in the gastrocnemius of the cat. These two peptides were not separable by the earlier method (8, 18).

The 150-cm. column operated at 30°

and 50° (Figure 9, *A*) is also useful in some studies on hydrolyzates of proteins or peptides. For example, hydroxyproline is best determined on a column operated at 30°, under which conditions it is well separated from aspartic acid. Glutamine and asparagine, which may be present after hydrolysis of peptides by leucineaminopeptidase (1, 13), can be differentiated from glutamic and aspartic acids at 30°. This separation has been very useful in studies of Hirs, Stein, and Moore (2) on the positions of the asparagine and glutamine residues in ribonuclease.

DISCUSSION

This discussion bears primarily upon specific problems encountered in the development of the present instrument. Some of the experience gained, however, is generally applicable to the design of equipment for automatically recording the results of a continuous colorimetric analysis of an effluent stream.

Flow Rate through Column. The rate of 30 ml. per hour adopted in the present work represents a compromise between the need for speed in the analysis and the desire for simplicity in the construction of the equipment. Presumably, ion exchange columns could be operated at higher rates of flow without loss of resolving power if resin possessing an even smaller particle size were used. The use of more finely divided resin would, however, necessitate operating pressures greater than the 4 atm. developed in the present apparatus, and this in turn might mean that conventional laboratory glassware would have to be abandoned in favor of equipment specially designed to resist higher pressures.

Control of Bubble Formation in Heating Coil. It is essential to the success of the present procedure that it be possible to heat the effluent-reagent mixture for 15 minutes at 100° without formation of bubbles of air or vapor. Air bubbles entering the absorption cell produce erratic absorbance readings, and bubbles once formed are hard to remove without mixing of the effluent stream. Bubble formation has been prevented, however, by the combined effects of three measures: use of deaerators in the buffer lines; application of a back-pressure equivalent to 10 cm. of mercury to the heating coil; and inclusion of detergent (BRIJ 35) in the buffers. In earlier experiments, the buffers were boiled and stored under oil. The introduction of the deaerators to remove air continuously from the solutions flowing to the pumps has proved simpler and more efficient. Air and nitrogen are both much less soluble in 75% methyl Cellosolve than in water, and, therefore, it is not necessary to remove dissolved gas from the ninhydrin solution. The tendency for bubbles to

form from the small amount of dissolved gas in the reagent is reduced by the existence of a back-pressure applied to the heating coil through the drain tube. The presence of detergent promotes superheating, and hence has the same effect. It is also important to exclude from the heating coil any foreign particles that might act like boiling chips. The glass wool filters in the ninhydrin line are included for this purpose. The smooth inner surface of the extruded Teflon tubing is doubtless also an advantage.

Avoidance of Mixing during Color Development and Photometry. For continuous photometry to be practicable, it is essential that the separations achieved by the chromatographic column not be vitiated by mixing of the effluent after the solution leaves the column. To evaluate the efficacy of various modifications in design, the resolution obtained when a given column was operated on a fraction collector at 30 ml. per hour was employed as a standard for comparison. The resolving power shown in Figure 2 is equivalent to that obtained when 0.5-ml. fractions are collected. To ensure such a degree of resolution, it was necessary to keep the diameter of the effluent stream sufficiently small. For example, beginning with a heating coil 2 mm. in inside diameter, progressive reduction of the bore to 0.7 mm. was required to obtain the desired results. In this narrow tubing, a 0.5-ml. aliquot of the effluent occupies a length of 150 cm. The bore of the absorption cell is similarly critical, appreciable mixing being encountered with tubes possessing an inner diameter greater than about 2.3 mm.

It is important to keep the glass tubing of the stopcock manifold to 1-mm. bore, and make the Teflon-to-glass connections without a section of wider diameter. Significant mixing does not occur in the pocket directly below the sintered plate of the chromatograph tube, as packing this space with fine glass beads does not improve the results.

Tests on Performance of Photometer. One of the first tests applied was designed to make sure that the absorption cell fitted sufficiently tightly against the slits of the adapters (Figure 5) to keep stray light from reaching the photocells. This was done by filling the cell with India ink and observing whether the recorder registered 0 μ a. (∞ absorbance) for each of the three cells. When thick-walled capillary tubing was used, this was not the case; the glass conducted light around the fluid in the bore.

Conformity to Beer's law was checked in three ways. First, with the top panel of the photometer removed, the cuvette holder of the upper cell was replaced with a holder for 18-mm. tubes. The blue color from a series of standard

solutions of leucine was measured, and a linear response was obtained up to and beyond an absorbance of 1.4, thus confirming the monochromatic transmittance of the filters as actually installed in the photometer. In the second test, solutions of increasing absorbance were drawn up into the 2.2-mm. absorption cell, and it was found that even at these higher concentrations of the blue color, Beer's law was obeyed up to and beyond an absorbance of 1.4. Finally, the recoveries obtained (Table II) when the load on the columns was varied over a 64-fold range established the adequacy of the performance under operating conditions. These data also prove that Beer's law is obeyed even at concentrations of blue color sufficiently high to give an absorbance of 1.4 in cell 3, which contains the glass rod that reduces the effective depth to about 0.7 mm.

Because the response curve (current output *vs.* illumination) of a photocell varies with the external resistance in the circuit, it was necessary to determine how much change could be made in the Helipot settings without introducing a significant change in the calibration of the instrument. By interposing neutral density filters or wire screen between the lens and the interference filter it was possible to simulate the reduction in light intensity that occurs during aging of the light bulbs. As long as the change in Helipot settings required to zero the recorder did not exceed 300 ohms, correct recoveries were obtained when a known mixture of amino acids was analyzed. These experiments form the basis for the statement that the light bulbs should be changed when the Helipot settings have been increased three turns above their initial value.

Ninhydrin Reagent. The difference in the composition of the ninhydrin reagent used in the present procedure from that in the fraction collector method is necessitated by the fact that the effluent-ninhydrin reagent mixture is not diluted before being read in the recording photometer. If the amount of hydrindantin used in the manual method is employed in the recorder, some of it will crystallize out as the solution cools after leaving the boiling water bath. Hydrindantin, though soluble in 75% methyl Cellosolve, is very sparingly soluble at room temperature in the 2 to 1 effluent-reagent mixture that contains only 25% methyl Cellosolve. In the fraction collector method, dilution of the reaction mixture with 50% ethanol keeps the excess of hydrindantin in solution. The dilution step has not been incorporated in the recorder method because of mixing problems.

Fortunately, it is feasible to avoid precipitation and still obtain satisfactory results by reducing the hydrindantin concentration. This is possible because deaeration considerably decreases

the requirement for hydrindantin, which is present in large excess (3 grams per liter) in the manual method in order to take care of the dissolved oxygen in the samples analyzed (9). A reagent containing 0.55 gram per liter of hydrindantin (anhydrous) was used initially with the recorder; this concentration is just below that which will cause precipitation. Experience has shown, however, that reagents prepared with stannous chloride (6) are more stable. Because stability is highly desirable, a reagent has been adopted that contains 0.4 gram of stannous chloride dihydrate per liter and can be stored for at least a month without deterioration. The presence of stannous chloride would cause a slight precipitate to form if phosphate buffers were employed (9), but this disadvantage is not of consequence with the buffer systems currently being used with the recorder. If necessary, a reagent containing 0.55 gram of hydrindantin can be adopted. It is stable for about 2 weeks, at the end of which time a reduction in color yields of about 4% can be anticipated.

As the amount of reduced ninhydrin present in the reagent must be limited in order to avoid precipitation, the color yields obtained with either the stannous chloride or the hydrindantin reagent are not maximal. The precise color yields are sensitive to the amount of reducing agent present, and therefore the stannous chloride or hydrindantin should be accurately weighed out (tared vessels are desirable for routine use).

The variations in the values of the constants listed in Table I are primarily a reflection of the differences in the color yields of the individual amino acids. The constants applicable to the present procedure were determined from the recorded effluent curves obtained upon chromatography of synthetic mixtures of amino acids. The fact that most amino acids are eluted quantitatively from Amberlite IR-120 has been established by the fraction collector method (5), in which independently determined color yields are used to calculate the recoveries. Exceptions are amino acids which decompose during chromatography, such as glutamine, for which the recovery is about 70%, and tryptophan, which is incompletely recovered from the 50-cm. column. The constants given in Table I correct approximately for the losses of these two amino acids.

The recorder provides a continuous record of the absorption at both 570 and 440 m μ . In peaks from most of the amino acids, the relative absorption at these two wave lengths is the same. Where there is a difference, the ratio of the absorptions at the two wave lengths can be useful as additional evidence for the identification of the compound responsible for the peak. For example, in

Figure 9 the relative amount of absorption at 440 m μ is high for glutamic acid, cystine, lysine, hydroxylysine, ornithine, and γ -aminobutyric acid.

Although the base line is normally steady, certain small and predictable variations are brought about as a consequence of the presence of phenol in the buffers. The treatment with 0.2N sodium hydroxide washes out most of the phenol, so that after the column is re-equilibrated with pH 3.25 buffer there is a slight rise in the 440-m μ base line at the retardation volume of the phenol. This rise usually occurs near the serine peak and does not interfere with the integration of proline, which emerges later. However, the rise may be close to a hydroxyproline peak in the 30°–50° system (Figure 9, A). If, therefore, the presence of hydroxyproline is anticipated, 200 ml. of buffer at pH 3.25 should be pumped through the 150-cm. column of the 30°–50° system before the sample is added. Phenol also causes a second small rise in the readings at 440 m μ at the point of breakthrough of the pH 4.25 buffer.

Adaptation of Instrument to Other Uses. The present apparatus has been used only for analytical purposes thus far. The materials analyzed, such as protein hydrolyzates, blood plasma, urine, and mammalian tissue extracts, have all been previously studied fairly thoroughly by the fraction collector method, and identification of most of the major constituents has been confirmed by paper chromatography and detailed chemical methods. Many amino acids encountered in research on plant and bacterial metabolism, however, have not been covered in the present studies. When the recorder is used to analyze types of mixtures not studied before, the position of a peak on the effluent curve frequently will not of itself furnish adequate identification. Under such circumstances, preparative scale columns may be needed to provide sufficient quantities of material to permit characterization of each component. It should readily be possible to use the recorder to monitor such columns by directing a small part of the eluent to the recorder while the major portion goes to a fraction collector. There is available on the recorder a marking pen which can be actuated by the fraction collector, so that the time of each fraction change can be marked on the recorder chart. When columns more than 1 cm. in diameter are used under several atmospheres of pressure, chromatograph tubes made of borosilicate glass pipe and possessing pressure fittings (Fischer and Porter Co.) have proved suitable.

The use of the recorder to study the chromatographic separation of the peptides in enzymatic hydrolyzates of proteins is probably feasible. The columns

of Dowex 50-X2, 0.9 cm. in diameter, used for this purpose can be operated with a pump at flow rates close to 30 ml. per hour (5).

Schram and Lombaert (11) have developed a scintillation counter which permits measurement and recording of the concentration of carbon-14 or sulfur-35 in a flowing stream without evaporation of the effluent. Their counting cell is designed to be inserted between a column and a fraction collector, but it could be interposed ahead of the stopcock manifold in the present instrument, to provide a continuous record of radioactivity in each of the peaks measured by the ninhydrin reaction.

ACKNOWLEDGMENT

The authors wish to express their appreciation to C. H. W. Hirs of this laboratory and Kenneth Woods of Cornell Medical Center, who have built and operated recorders according to this design, and have given the valuable benefit of their experience with the instrument. They also are indebted to Herbert Jaffe for the spectroscopic data on the filters, to Nils Jernberg and Carl R. Ti-

den of the Instrument Shop for expert advice and shop work on the photometer, and to Kerstin Johansson for skillful technical assistance. It is a pleasure to acknowledge the correspondence of D. H. Simmonds of Melbourne concerning the details of the instrument which he has designed to render the discontinuous type of procedure automatic (12).

The authors appreciate the suggestion of E. G. Pickels and J. E. Miller, Spinco Division, Beckman Instruments, Inc., regarding the "dot-counting" method of measuring the width of a peak, and are indebted to them for permission to describe the procedure.

The procedure for the accurate measurement of the rate of flow was suggested by A. J. P. Martin, to whom the authors are much indebted.

LITERATURE CITED

- (1) Hill, R. L., Smith, E. L., *J. Biol. Chem.* **228**, 577 (1957).
- (2) Hirs, C. H. W., Stein, W. H., Moore, S., IUPAC Symposium on Protein Structure, Wiley, New York, in press.
- (3) Hirs, C. H. W., Stein, W. H., Moore, S., *J. Biol. Chem.* **211**, 941 (1954).
- (4) *Ibid.*, **221**, 151 (1956).

- (5) Moore, S., Spackman, D. H., Stein, W. H., *ANAL. CHEM.*, **30**, 1185 (1958).
- (6) Moore, S., Stein, W. H., *J. Biol. Chem.* **176**, 367 (1948).
- (7) *Ibid.*, **192**, 663 (1951).
- (8) *Ibid.*, **211**, 893 (1954).
- (9) *Ibid.*, p. 907.
- (10) Schram, E., Dustin, J. P., Moore, S., Bigwood, E. J., *Anal. Chim. Acta* **9**, 149 (1953).
- (11) Schram, E., Lombaert, R., *Ibid.*, **17**, 417 (1957).
- (12) Simmonds, D. H., *ANAL. CHEM.* **30**, 1043 (1958).
- (13) Smith, E. L., Spackman, D. H., *J. Biol. Chem.* **212**, 271 (1955).
- (14) Soupart, P., Moore, S., Bigwood, E. J., *Ibid.*, **206**, 699 (1954).
- (15) Spackman, D. H., Stein, W. H., Moore, S., *Federation Proc.* **15**, 358 (1956).
- (16) Stein, W. H., *J. Biol. Chem.* **201**, 45 (1953).
- (17) Stein, W. H., Moore, S., *Ibid.*, **211**, 915 (1954).
- (18) Tallan, H. H., Moore, S., Stein, W. H., *Ibid.*, **211**, 927 (1954).
- (19) *Ibid.*, **230**, 707 (1958).

RECEIVED for review February 28, 1958. Accepted May 15, 1958. Presented in part before the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1956. The second and third instruments of this type were constructed with the aid of a grant from The National Science Foundation.

Separation of Isomeric Polyphenyls by Adsorption Chromatography

MAX HELLMAN, ROY L. ALEXANDER, Jr., and CHARLES F. COYLE

National Bureau of Standards, Washington 25, D. C.

► The chromatographic behavior of selected polyphenyls was studied to develop a method for separating mixtures of these compounds. A variety of adsorbents such as alumina, fuller's earth, silica gel, and different grades of charcoal were used. The separations of known mixtures were studied by the elution technique and elution curves were drawn. Results obtained with several adsorbent-eluent systems are compared. Some separation occurred in most cases, with the alumina-iso-octane combination providing maximum over-all effectiveness in separation and recovery. Some charcoals give better separation but material recovery is poor. On the basis of the results obtained, the behavior of mixtures of higher polyphenyls is predicted.

THE SEPARATION of mixtures of aromatic compounds by chromatographic techniques has found extensive use in recent years, particularly in the

analysis of heavy oils and tars. However, nearly all of the work done in the field deals with the separation of mono- and polynuclear aromatics.

The group of aromatic compounds known as polyphenyls, which correspond to the general formula, $C_6H_5(C_6H_4)_nC_6H_5$, has been almost entirely neglected. Only one paper in the recent literature (6) has dealt with the attempted separation and identification of mixtures of polyphenyls by paper chromatography. Recent interest in biphenyl and related compounds as potential high temperature lubricants and nuclear reactor coolant-moderators (3) has created a need for a systematic method of analysis of mixtures resulting from the thermal or radiative degradation of these materials. Consequently, this study was undertaken with the aim of obtaining some information concerning the behavior of polyphenyl mixtures on a variety of chromatographic adsorbents. No attempt was made to find ideal conditions for a specific separation, but rather

to establish definite trends which would enable one to choose the proper system for a given mixture.

REAGENTS

Adsorbents. Alumina, Fisher Scientific Co., absorption grade, 80 to 200 mesh. Reactivated by heating at 200° C.

Fuller's earth, Floridin Co., Florex XXF. This was sifted and 100- to 200-mesh size was selected. Reactivated by heating at 160° C.

Silica gel, Davison Chemical Co., Code 912, 28 to 200 mesh.

Pittsburgh charcoal, Pittsburgh Coke and Chemical Co., 8 to 30 mesh.

Coconut charcoal, National Carbon Co.

Bone char, Baugh & Sons Co.

The above charcoals were ground and 40 to 60 mesh was employed. Each charcoal was heated at 110° C. for several hours prior to usage.

Deactivation of alumina, silica, and Pittsburgh and coconut charcoal was performed by mixing the desired amount of deactivation agent (usually water) with the adsorbent in a glass-stoppered