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Gilbert

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[54] **APPARATUS FOR SEQUENCING PEPTIDES AND PROTEINS**

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[73] Assignee: **Beckman Instruments, Inc.,** Fullerton, Calif.

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[52] **U.S. Cl.** ..... **23/253 R**

[51] **Int. Cl.** **G01n 33/16**

[58] **Field of Search** ..... 23/253 R, 259, 253 PC

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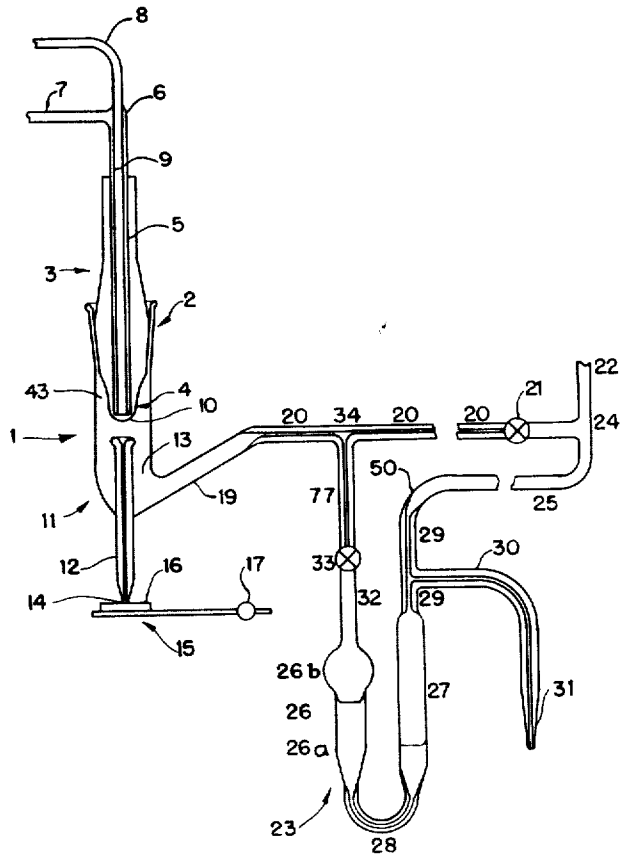
Attorney, Agent, or Firm—R. J. Steinmeyer; F. L. Mehlhoff

[57] **ABSTRACT**

A sequencer which includes a reaction chamber having inlet and outlet ports, a sample holder of variable temperature adapted to extend downwardly into said chamber and serving to retain a sample as a film on its outer surface, a plurality of chemical reservoirs maintained at controlled temperature and means for selectively introducing into said chamber chemical vapors from said reservoirs to condense upon said holder and selectively react with the sample and for introducing inert gas to evaporate the chemicals from the holder.

20 Claims, 12 Drawing Figures



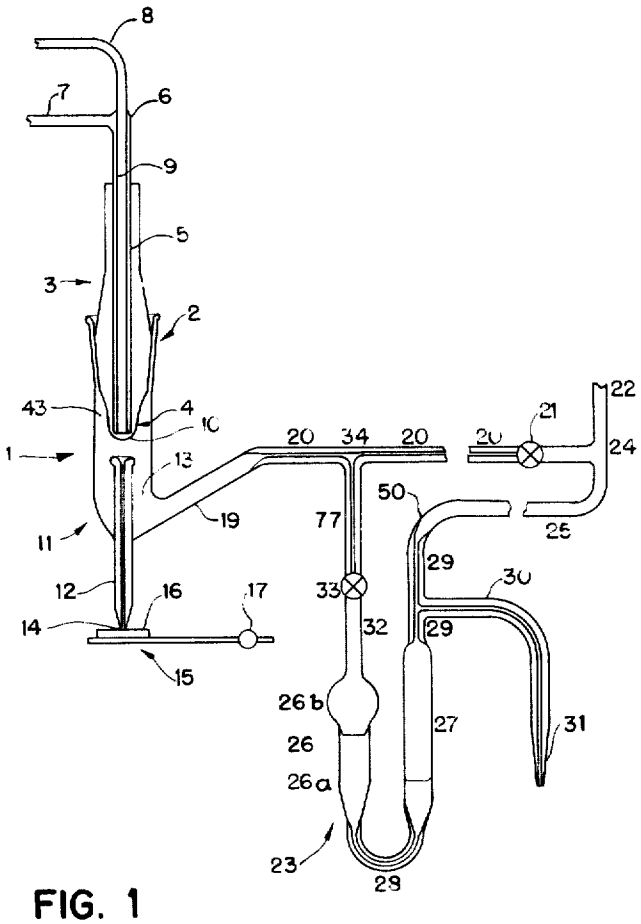


FIG. 1

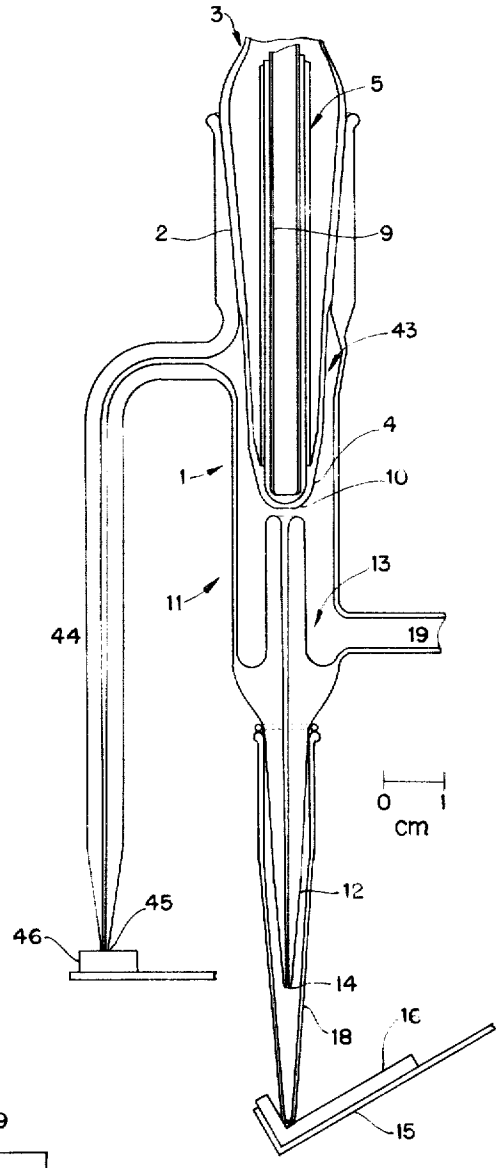


FIG. 3

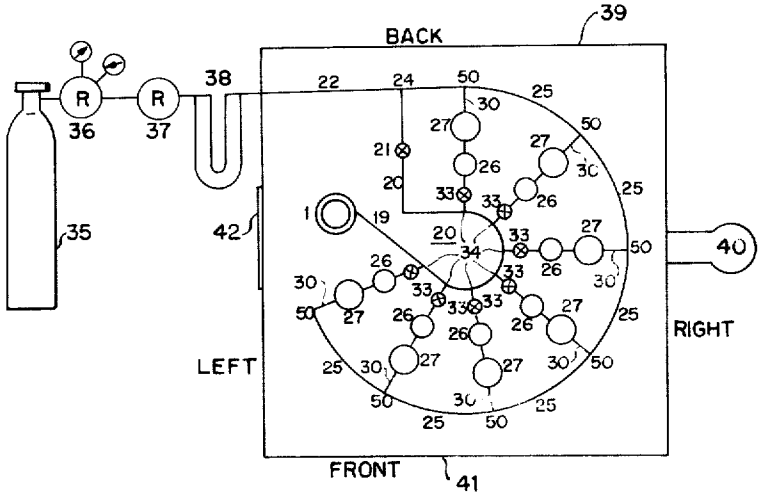


FIG. 2

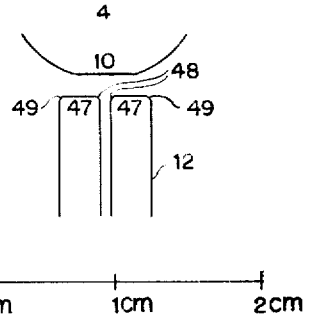


FIG. 4

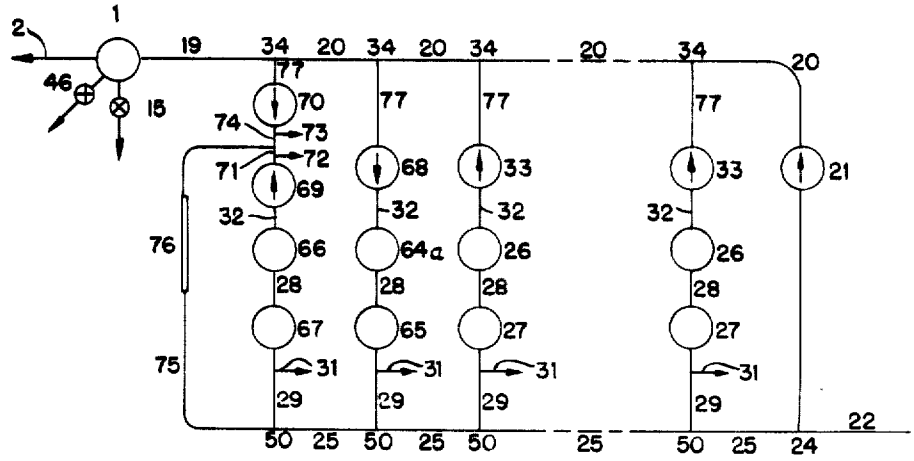


FIG. 6

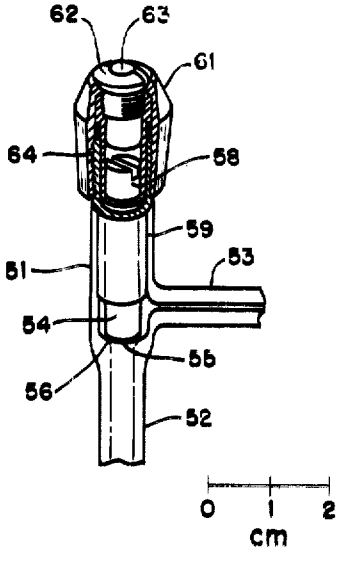


FIG. 5

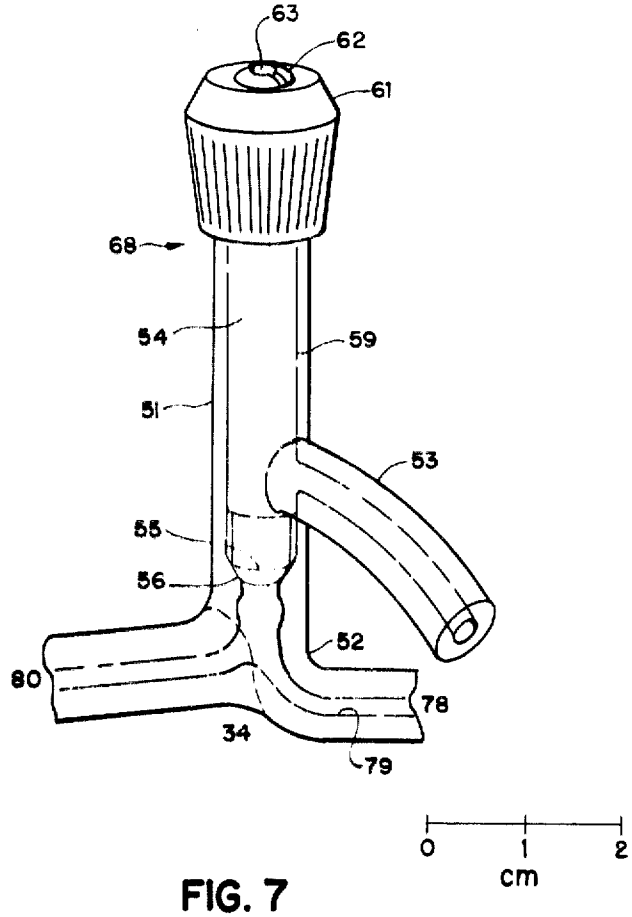
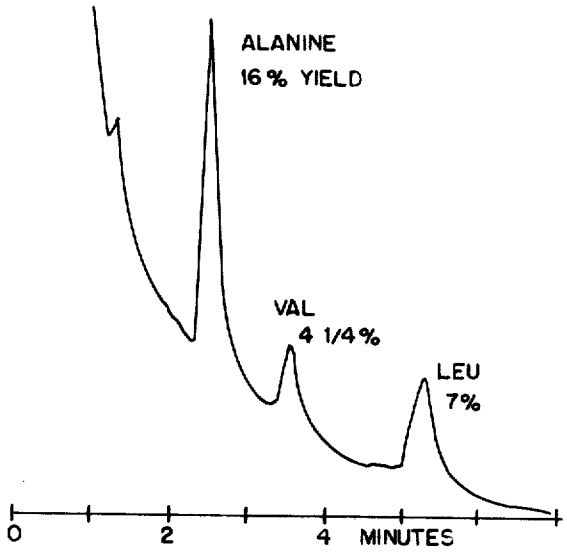


FIG. 7

FIG. 8 A



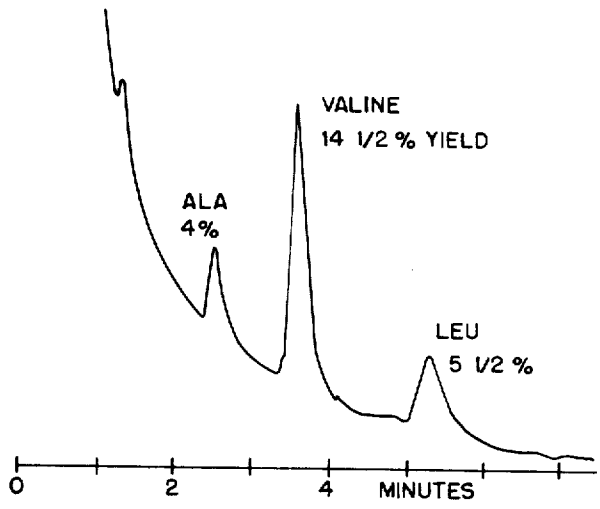


FIG. 8 B

FIG. 9 A

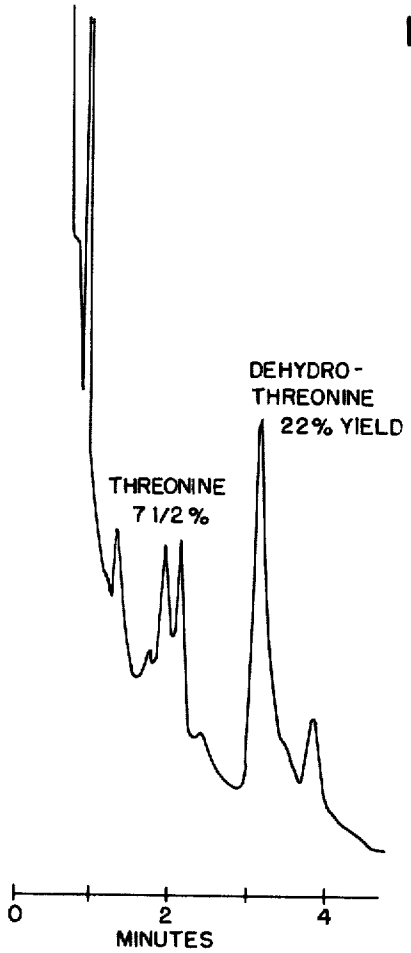


FIG. 9 B

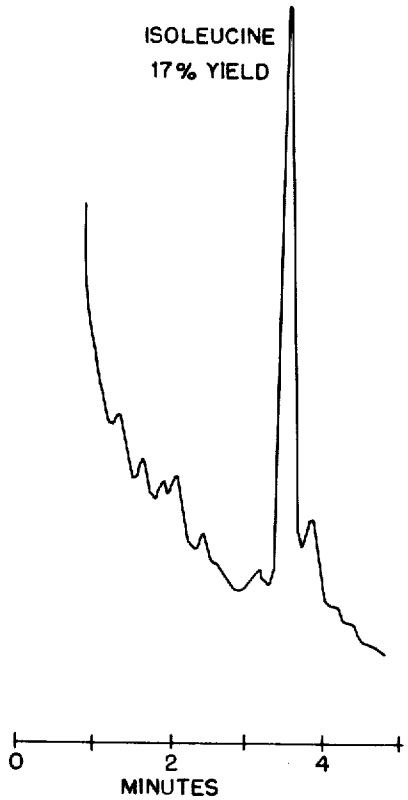


FIG. 9 C

APPARATUS FOR SEQUENCING PEPTIDES AND PROTEINS

BACKGROUND OF THE INVENTION

This invention relates generally to an apparatus for sequencing small quantities of proteins and peptides.

An analytical procedure, known as the Edman degradation, has been developed for determining the sequence of amino acids in proteins and peptides. The degradation or removal of the terminal amino acid residue from the protein chain takes place in two steps, coupling and cleavage, represented by the following consecutive reactions:

$$Q{-}NCS + H_2N{-}CHR{-}CO{-}NH{-}CHR'{-}\ldots \underset{-COOH \rightarrow}{}$$

$$Q{-}NH{-}CS{-}NH{-}CHR{-}CO{-}NH{-}CHR'{-}\ldots \underset{-COOH \rightarrow}{}$$

$$Q{-}NH{-}\underline{C{=}N{-}CHR{-}CO{-}S} + H_2N{-}CHR'{-}\ldots \underset{-COOH.}{}$$

Here Q—NCS is an isothiocyanate, commonly phenyl isothiocyanate, in which Q would be the phenyl group, C_6H_5. The molecule of the protein sample is shown with the amino terminus at the left and the carboxy terminus at the right. The amino-terminal amino acid is $H_2N{-}CHR{-}COOH$ and the penultimate amino acid is $H_2N{-}CHR'{-}COOH$. R and R′ represent the side-chains that distinguish the amino acids. An amino acid "residue" is a grouping of the form —NH—CHR—CO—, equivalent to an amino acid minus water. The protein consists of a chain of residues having a complete H_2N-group at one end and a —COOH group at the other. The isothiocyanate simply combines with the protein, forming the (phenyl)thiocarbamyl-protein. This reaction, known as coupling, takes place in a buffer of pH about 9 that can dissolve both the isothiocyanate and the protein, and at a temperature of typically 50°C. After removal of the buffer and excess reagent, the thiocarbamyl-protein is treated with an anhydrous acid, which causes it to split between the terminal and penultimate residues. This reaction, known as cleavage, leaves the original protein unchanged except for the loss of its terminal amino acid residue. The portion split off cyclizes to form (when phenyl isothiocyanate is used) an anilinothiazolinone

$$Q{-}NH{-}\underline{C{=}N{-}CHR{-}CO{-}S}.$$

This is subsequently isomerized by a reaction known as conversion, which is carried out separately and not in the sequencing apparatus, to a (phenyl) thiohydantoin (PTH) of the amino acid. The PTH is finally identified by thin-layer chromatography or now, more commonly, by gas chromatography. The shortened protein is next subjected to a second cycle of degradation, and so forth.

An apparatus for automatically sequencing proteins and peptides was described in the European Journal of Biochemistry 1, pages 80–91, in 1967. Since then several commercial sequencers have been developed. In these sequencers, a sample is contained in a spinning cup where it spreads into a thin film upon its vertical cylindrical wall due to the centrifugal forces. The cup is contained in a small heated chamber connected to a source of inert gas and to a vacuum system. Reagents and solvents are stored in reservoirs and delivered through small tubes by pressure of inert gas to the bottom of the cup where they climb the walls due to the centrifugal forces and mix with the sample film. The quantity delivered is controlled by the net pressure and open time of the delivery valves. Extracts resulting from the passage of solvents over the protein film are collected at the top of the cup by means of a tube that acts as a scoop. The extracted liquid is diverted to waste or to a fraction container for subsequent analysis. To dry the sample, the chamber is evacuated.

The values for controlling the liquids, the vapors and the vacuum demand very careful design to avoid contamination through leakage, absorption, permeation or corrosion. In a successful protein sequencer, there is a multiplicity of pressure regulators, manifolds, controlled leaks, purging means, means for automatically controlling certain valves, means for controlling the rate of evacuation, vacuum gauges and seals and the like. The spinning cup requires a special bearing, drive and speed control. Thus, the prior art sequencers are relatively complex and expensive.

A cycle of degradation in a sequencer of the above type is typically carried out as follows: The coupling buffer and a solution of isothiocyanate are delivered in precisely controlled amounts to the cup, where they spread over the protein sample. The sample dissolves in the liquid layer and undergoes coupling. After a specified reaction time, for example, 30 minutes at 50°C., a graduated vacuum is applied to remove the volatile substances. The coupled protein is then washed free from the unevaporated reagents and byproducts by passage of solvents, benzene followed by ethyl acetate, over it and out through the scoop and waste tube. The sample is again dried by evacuation. The cleaving acid is then added in a precisely controlled amount and left in contact with the sample. The cleaving acid is next removed by evacuation, and the thiazolinone is finally extracted by a suitable solvent flowing up over the sample and out through the scoop to the fraction container. After drying by evacuation, the sample is ready for the next cycle of degradation. The collected thiazolinone is stored for subsequent conversion and identification at the convenience of the operator. The operating details of a cycle are more intricate than the above outline suggests and can be modified to suit the individual sample. A cycle typically requires from 1½ to 2 hours.

The time required, the quantities of sample required and the amount of chemicals consumed are governed in large measure by the dimensions of the apparatus and the interplay of the forces involved, viz., centrifugal, adhesion, wetting, surface tension and the like, and the rates at which the basic operations, viz., mixing, dissolution, evaporation and extraction, can take place in sample films having the dimensions dictated. It has not yet been found feasible to speed the cycle of operation or diminish the sample size to any considerable extent.

Generally, due to the above factors, the sequencer requires samples of 300 nanomoles or more for a successful long run and as much as 1 micromole for best performance with shorter peptides. Months of effort may be required to collect and purify such quantities of protein samples. The investigator reluctantly commits the entire amount of his material to a sequencer for analysis.

The numerous valves, pressure regulators, plastic tubes and other components hamper maintenance of a sufficiently pure atmosphere, slow the rate of evacua-

tion and encourage mutual contamination of chemicals at the various stages of the degradation cycle.

One troublesome phenomenon in the use of volatile coupling buffers is the formation of so-called "dew", an involatile liquid deposit consisting of the salt formed by reaction of the buffer amine with the cleavage acid. Dew formation is self-accelerating in that the dew formed serves as a sink for the next increment. The dew, as well as all plastic components exposed to the chemicals or their vapors, acts as a sink or reservoir for the vapors and subsequently releases them to contaminate the sample, reagents and inert atmosphere.

The yield of the expected terminal amino acid obtained at each cycle, called the specific yield, diminishes through successive cycles due to impurities, cross-contamination, and other factors. Yields of other amino acids, resulting from asynchrony or non-specific cleavage or both, increase relative to the specific yield. Asynchrony arises when some of the molecules of the sample get out of step as the sequencing advances; and nonspecific cleavage is a breakage of peptide bonds other than the intended terminal bond. These non-specific yields, together with byproduct impurities, interfere with the identification of the specific amino acids and ultimately become so great that there is no use of continuing a particular run.

Another problem with the prior art apparatus is that large quantities of reagents and solvents are required. Since they must be exceptionally pure, purification and testing of chemicals becomes exceedingly costly.

The sequencer itself is large, complex and costly. Thus, it is not within the means of a large number of protein chemists. Because of its complexity, it cannot be repaired by the chemist but must generally be repaired by highly trained field engineers.

OBJECTS AND SUMMARY OF THE INVENTION

It is a general object of the present invention to provide a relatively simple, compact, inexpensive sequencer for proteins and peptides.

It is another object of the present invention to provide a sequencer in which the time required for each cycle of degradation is minimized while the efficiency of degradation is increased to permit more extended sequencing.

It is a further object of the present invention to provide an apparatus in which asynchrony and non-specific cleavage are minimized.

It is a still further object of the present invention to provide a sequencer which, because of its mode of operation and size, reduces the amount of sample and chemicals required to carry out a sequencing operation.

It is a further object of the present invention to provide a sequencer which minimizes absorption of vapors and the formation of dew.

The foregoing and other objects of the present invention are achieved by a sequencer in which the protein sample to be sequenced is supported in a thin layer by natural forces of adhesion upon the lower tip of a downwardly extending temperature controlled sample holder or member disposed in a reaction chamber and a plurality of chemical reservoirs also at controlled temperature communicate with the reaction chamber. Inert gas is bubbled through selected chemical reservoirs as needed and the chemicals are transported as vapors by the inert gas to the reaction chamber to condense on the sample to carry out the degradation process in a sequence of steps substantially in accordance with those described.

More particularly, a protein sample weighing a few micrograms adheres as a thin film to the end of the sample holder which can be heated or cooled from within and which is mounted within a small reaction chamber having entrance and exit ports. The components of the coupling buffer and the isothiocyanate, carried as vapors from heated reservoirs through which inert gas bubbles, are condensed upon the cooled holder where they remain as a hanging drop. The chamber is closed, the holder is warmed and the protein dissolves in the liquid, and coupling proceeds. When coupling is completed, inert gas is passed over the holder to evaporate off the chemicals. The holder is next cooled, cleaving acid is carried as a vapor and condensed upon the holder, the chamber is closed, and the holder is again warmed to let cleavage occur. The acid is evaporated, the holder is cooled, and solvent vapor is condensed upon the holder in sufficient amount to drop off the end. The extract runs down a drain tube and is collected for later conversion and identification. The sample is rewarmed and dried and is then ready for the next degradation cycle.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a schematic view of a portion of a sequencer assembly in accordance with the invention showing the reaction chamber, sample holder, drain and related vapor and gas supply conduits.

FIG. 2 is a plan view of a sequencer assembly.

FIG. 3 is a view of a preferred embodiment of the reaction chamber, sample holder and drain.

FIG. 4 is an enlarged view of the lower portion of the sample holder and upper portion of the drain tube.

FIG. 5 is a view, partly in section, showing a suitable valve for use in connection with the sequencer assembly.

FIG. 6 shows schematically the gas and vapor flow through the manifolds and particularly the method of handling acids and amines.

FIG. 7 shows an improved connection between the valve of FIG. 5 and the outlet manifold.

FIGS. 8A and 8B show gas chromatograms of 40% aliquots of the 15th fraction (alanine) and the 17th fraction (valine) collected in an 18 cycle degradation of a 5 nanomole sample of myoglobin.

FIGS. 9A, 9B and 9C show gas chromatograms of 40% aliquots of the seventh fraction (cysteine), eighth fraction (threonine) and 10th fraction (isoleucine) of an 18 cycle run of a 5 nanomole sample of insulin A.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A schematic view of a portion of the sequencer is shown in FIGS. 1 and 2. The apparatus which is preferably constructed of borosilicate glass includes a reaction chamber 1 having a small interior volume, typically 3 ml. or less. The chamber is built upon a conical ground tapered joint 2. A male member 3 is adapted to fit into the joint and includes a closed finger-shaped extension forming a sample holder 4 extending below the ground surface into the reaction chamber. The sample holder 4 is typically 1 cm. in diameter or less. The sample holder 4 is joined internally to a tube 5 that passes axially up through the male member 3. The outer part

of the male member could itself serve as such a tube, but better thermal insulation is achieved by joining the smaller tube **5** to the tip of the sample holder **4**, leaving an air space between the upper part of member **3** and tube **5**. Tube **5** is connected by a seal **6** to a side arm **7**. A second tube **9** passes concentrically within tube **5** to a point close to the tip of the holder where it terminates.

The sample is applied conveniently as a solution in the amount of a few microliters to the tip **10** of the sample holder to which it clings by adhesion. The temperature of the sample holder and the sample is controlled by circulating warm or cold water through the holder. The water enters, for example, through the side tube **7**, flows down through the annulus between the tubes **5** and **9** and impinges upon the inside surface of the tip **10** to which it imparts its temperature and then returns through the tube **9**, passing out through the extension **8**.

During operation, the male member comprising the holder **4** and the water circulating system just described, which can be removed as a unit, is mated with the chamber **1**. The chamber proper communicates with at least two conduits. One of these conduits is a vertical drain **12** with a capillary bore typically 1 mm. or less, situated directly below the holder **4** and passing through the bottom of the chamber **1**. Its upper end may or may not extend above the bottom of the chamber. In the example shown, it extends upwardly into the chamber leaving an annular space **13** between it and the wall of the chamber. This space promotes uniform distribution of the entering vapors.

The top of the drain **12** is shaped to receive drops of liquid falling from the lower end of the holder so that they may pass down the drain and out of its lower tip **14**. The lower part of the drain is relatively long so that it reaches well down into a fraction container **18** (see FIG. 3) with which it is mated. During reaction, the chamber **1** is closed while at other times, such as during collection, the chamber is open. To close the chamber, a simple valve **15** can be mounted below the drain. The valve, for example, may comprise a rubber pad **16** mounted upon an arm pivoted about an axis **17**. The arm may be raised or lowered by suitable means to seal the end of the downwardly extending drain tube. Because the lumen of the tip **14** is very small, typically much less than 1 sq. mm., the presence of rubber in contact with the space within the chamber **1** has a negligible effect upon the purity of the atmosphere or reacting mixture within the chamber.

The second tube connected to the chamber is vapor tube **19** which, in the example shown, enters the bottom of the chamber from the side, next to the junction of the drain **12** with the reaction chamber **1**. The tube **19** is connected to tube **20**. The tube **20** serves as the outlet manifold for a series of junctions and valves which selectively convey inert gas laden with chemical vapors or pure inert gas to the tube **20** and reaction chamber during a degradation cycle. The tube **20** is preferably of a small bore (e.g. 1 mm.) to minimize the volume in communication with the chamber. The tube **20** is connected by valve **21** to the tube **22** to which a mildly pressurized inert gas such as pure nitrogen at a pressure of about 25 millibars is supplied. It will be understood that during flow of gas through valve **21**, the gas passes successively through the tube **22**, valve **21**, and tubes **20** and **19** into the chamber **1** and out through the drain **12** to escape through open valve **15**. With this in mind, we may speak of upstream and downstream portions of the apparatus.

A multiplicity of reservoir assemblies, of which one is schematically shown in FIG. **1** generally at **23**, are connected in parallel to the inlet manifold **25**, which is an extension of tube **22** beyond the junction **24** at which valve **21** is connected to tube **22**. In a typical embodiment, there may be as many as seven such reservoir assemblies all more or less alike in construction except that some may have larger volumes than others. Inlet manifold **25** conveys inert gas to each of the reservoirs as needed.

Each reservoir assembly **23** comprises a main reservoir **26** and an auxiliary reservoir **27** known as a backup bulb, the latter being upstream from the former. The lower ends of the two reservoirs **26** and **27** are joined by a short length of capillary-bore tubing **28**. The upstream end of the backup bulb **27** is joined to inlet manifold **25** by a length of capillary bore tubing **29** to which, at some suitable distance from the junction **50** of tubes **29** and **25**, is joined a side tube **30** having a constricted orifice or terminal portion **31** through which, under the pressure prevailing in the inlet manifold **25**, the inert gas leaks to the outside at a slow rate, typically a few milliliters per minute. The purpose of this leak will be presently described but in essence it is to prevent backflow of any vapors into the inlet system where they might contaminate vapors in adjacent reservoirs. The downstream or upper end **26*b*** of the reservoir **26** is connected through a suitable length of unconstricted tubing **32** to valve **33** which may be of a design to be presently described comprising a core of wholly inert material such as polytetrafluoroethylene (PTFE) seating and sealing against a borosilicate glass body. This valve is merely an on-off valve requiring no control of flow rate. The valve **33** is, in turn, connected on its downstream side to the outlet manifold **20** at a compact tee joint **34**.

Each reservoir assembly **23** contains a single pure liquid such as butyl chloride, water, etc., needed for carrying out the process of Edman degradation. The liquid is conveniently introduced into the reservoir by removing the core of the valve **33** to permit entry of a pipette, funnel or tube through which the liquid is poured or injected from a stock container into the reservoir **26**. When the valve **33** is reassembled, the system is sealed from outside air except for the leak **31**, the drain **12** when open, and the ground joint **2** which may leak slowly, and through which inert gas or vapors pass only in an outward direction. When the system is properly operated, no outside air can enter into the system.

Once contained in the reservoir **26**, the liquid is free to move between the reservoir **26** and the backup bulb **27** under the influence of pressure of gas or vapor that it experiences. The quantity of liquid is such that it cannot overfill either the reservoir **26** or the bulb **27**. The reservoirs and backup bulbs intended for the components of the coupling mixture and for the cleaving acid are usually no larger than 5 ml. in capacity, while those intended for extracting solvents may have a capacity of 25 ml. each. The small reservoirs consist desirably of a lower cylindrical portion **26*a*** to provide an adequate depth of liquid for the bubbles to rise through and an upper expanded portion **26*b*** within which the surface of the liquid will normally lie during bubbling and which helps to break films and to discourage entrain-

ment of liquid that might otherwise reach the valve **33.**

When a chemical is to be delivered to the protein sample adhering to the tip **10** of the holder, valves **33** and **15** are opened while the valve **21** is closed. The pressure of the inert gas is then exerted across the liquid in the reservoir **26, 27.** Liquid rises in reservoir **26** until bulb **27** is empty and the gas then bubbles up through the liquid in the reservoir **26** and becomes nearly saturated with vapor. The vapor-laden gas passes through the valve **33,** the outlet manifold **20** and vapor tube **19** into the annular space **13** in the reaction chamber, through the gap between the holder **4** and drain **12** and out through the drain.

All parts of the apparatus described up to this point are held at an elevated temperature suitable for carrying out the reactions. Consequently, if the holder is cooled by circulation of cold water, that is, water at a temperature lower than that of the liquid chemical, the vapor of the chemical will condense on the holder and wet the protein sample. The gas, partially or largely depleted of its burden of vapor, passes out through the drain **12.**

FIG. **2** shows the entire apparatus in a schematic form. A tank of compressed inert gas **35** supplies the sequencing apparatus with inert gas through a high pressure regulator **36** and low pressure regulator **37.** The inert gas passes through a container of manganous oxide **38** which removes traces of oxygen. The manganous oxide, which changes from green to brown on oxidation, can be regenerated when necessary by heating it in hydrogen. The container **38,** made of borosilicate glass, is sealed directly to tube **22** to prevent permeation of air into the apparatus.

A container, box or housing **39,** typically a 1-foot cube in size and shape, serves to enclose the entire apparatus downstream of the manganous oxide container. The walls, top and bottom of the housing **39** are made of thermally insulating material, except for one side, the front, which consists of a glass window **41** to permit viewing the sequencer. This window is usually covered by an insulating cover to prevent thermal gradients. It is uncovered only briefly to view the sequencer. The interior of the housing **39** is kept at a uniform temperature, for example, between 55° and 65°C. In order to prevent distillation of the reagents from the sample holder **4** during coupling and cleavage, the housing temperature must exceed by a few degrees, typically 5°C., the reaction temperature imparted to the holder. The housing can be kept warm by suitable means, such as an electric hot air blower **40** mounted near the bottom at one side of the housing. Satisfactory thermal uniformity within a range of 1° over all parts of the apparatus has been obtained by attaching a branch conduit to the blower which directs hot air against the lower central area of the window **41** and another branch conduit which directs air against the area of the door **42** opposite the blower **40.** The small door **42** gives access to the reaction chamber **1** for the purpose of applying and removing the fraction container **18.** The top of the housing is removable to permit servicing the apparatus, filling the reservoirs and loading the sample upon the holder. The roof is perforated with holes through which protrude the water tubes **6, 7** and **8** and rods or handles for manipulating the various valves.

In the embodiment shown in FIG. **2,** the inlet manifold **25** is shown as a long curved tube occupying the upper region of the housing. The first branch of the tube is that at **24** supplying the valve **21.** Downstream from branch **24** is a series of branches **50,** each supplying a reservoir assembly **27, 26** and side tubes **30** serving as leaks. The reservoirs **26** with their valves **33** are connected at a corresponding set of joints **34** to the outlet manifold tube **20** which forms a rough circle within the larger circle of the inlet manifold **25.** The outlet manifold terminates as vapor tube **19** connected to the chamber **1.**

The interconnected glass assembly is relatively rugged while the various interconnecting parts are fairly flexible. The tubes **28,** being the lowest part of the apparatus, are adapted to rest on the floor of the housing which may be of soft material such as polyurethane foam and provides a yielding support for the apparatus for absorbing stresses imposed by uneven construction or inadvertent motion.

The chamber **1,** as shown in FIG. **1,** contains an upper annular space **43** between the holder **4** and the upper walls of the chamber **1.** When gas is passed through the chamber, entering from the vapor tube **19** and leaving the drain **12,** for the purpose of drying the sample on the tip **10** of the holder, it tends to bypass the space **43.** The gas in this space may, therefore, become somewhat stagnant and may retain vapors that contaminate the next reaction mixture. To expel such vapors, i.e., to purge the space **43** and ventilate the chamber **1** thoroughly during a drying step, I have found it desirable to add an upper vent **44** as shown in FIG. **3.** FIG. **3** is a preferred embodiment of the reaction chamber. The vent tube **44** has a capillary bore to keep the volume of the space in contact with the chamber to a minimum, but not so small as to greatly restrict the flow of gas. Its tip **45** is smooth and thin-walled and is closed by a rubber pad **46** on a valve arm like that of valve **15.** In drying the sample, it is advantageous to open only the drain valve first until most of the liquid has evaporated. The upper vent valve is then opened only briefly to purge the chamber.

The exit ports, drain **12** and vent **44,** permit controlling the flow rate of inert gas for condensation of chemicals and for drying. Typical operating pressures of inert gas in the inlet manifold **25,** as delivered by the regulator **37,** is 25 millibars. This is enough to overcome the head of liquid in the reservoir **26** and the back pressure due to surface tension of the meniscus in the tubes **28** and to provide a lively rate of bubbling. The rate must be high enough so that the time needed for condensation of chemicals is not unduly long, but not so high as to carry liquids past the valves **33.** Besides, the efficiency of evaporation and condensation of the liquid falls at higher bubbling rates so that beyond a certain flow rate there is little further gain in speed of transport of vapor to the tip **10** of the holder. The flow rate could be controlled by the degree of opening of valve **33** but this would require accurate adjustment. Instead, the valve is opened wide enough to offer no significant resistance to gas flow and control is achieved by constricting the bore of the drain **12.** During bubbling, when the drain is open and the vent **44** closed, at least half of the total pressure drop is across the drain **12.**

When the vent **44** is open, the flow of gas through the chamber is increased typically fivefold above that provided by drain **12.** Such high flow rates are too great for efficient vapor transport and are incompatible with the

dripping process, as will be described. Vent **44** is, therefore, used primarily for purging of the chamber as mentioned above. Both its location and its lower flow resistance greatly speed the ventilation of the chamber and thus save time. It might be supposed that for fast drying, vent **44** should be opened at the start of the drying step. However, when the vent is open, there is little pressure drop through the drain **12.** Consequently, less gas sweeps through the gap between the tip of holder **10** and the top of the drain **12** when the vent is open than when only the drain is open. Therefore, it is preferable to open the vent only towards the end of the drying step.

Another factor which governs the choice of gas flow rate is the mechanism of dripping from the tip of the holder. The tip **10** is advantageously ground to a rougher, flat surface having a circular area of a few mm. in diameter as shown in FIGS. **3** and **4.** Such a rough flat tip promotes uniform distribution of the sample and minimizes agglomeration into small thick masses. However, during the collection of extracts, drops formed at such a tip tend to be rather large and to have a rather low "dropping fraction". The dropping fraction is the fraction of the total liquid adhering to the tip that falls off in each drop. By way of example, ethyl acetate dripping from a holder such as that shown in FIG. **3,** but with the drain farther below the holder, may fall in drops of 26 microliters volume with a dropping fraction of 0.55. Such a low fraction makes for inefficient extraction and such a large volume contributes to loss of short peptides. I have found a means of correcting this condition, obtaining smaller drops at higher dropping fraction, without changing the form of the holder. This is accomplished by proper placement and proper design of the top of the drain tube **12.** The optimal arrangement is shown in FIG. **3** and enlarged in FIG. **4.** The top of the drain **47,** typically a capillary tube of 6 mm. outside diameter and 1 mm. inside diameter, is cut flat and square and is fire-polished so that at least the edges of the lumen **48** are rounded. In addition, the gap between the tip **10** of the holder and the top **47** of the drain is carefully adjusted. The optimum gap is found by carrying out actual dripping tests under various conditions with the liquids commonly used as solvents in the Edman degradation process, for example, ethyl acetate and butyl chloride. In the case of the design of FIGS. **3** and **4,** with the flat part **10** of the holder having a diameter of 4 mm., I have found a gap of 1.4 mm. to be about right. With such a gap, the drop volume will be about 13 microliters, that is, half what it would be were the drain farther below the holder; and the dropping fraction will be at least 0.7, depending on the wetness of the liquid. Ethyl acetate, for example, wets glass more readily than butyl chloride and drains more efficiently down the sides of the holder. With ethyl acetate, the dropping fraction may be as high as 0.9. If the gap is too large, the drops become larger and the dropping fraction smaller. In fact, there is a gap at which the dropping fraction is minimal. If the gap is too small, the drops will not fall free. Instead, the liquid tends to bridge the gap, remaining in contact with both the holder and drain and failing to detach itself from the holder. Ideally, the drop on growing large enough to touch the drain will momentarily bridge the gap and then come free of the holder, running down the drain. Despite the flat top of the drain, liquid does not run over the outer edge **49.** It all goes down the lumen. This action is, of course, encouraged by the flow of gas passing down through the drain.

It is important for proper dripping of the extracts that the flow rate of inert gas be relatively low. Since a typical extract is of a very small volume, such as 50 microliters, it should be efficiently swept through the drain into the fraction container to avoid loss of material. For proper drainage, a flow of gas through the drain is needed and consequently only the drain **12** is opened during extraction because its resistance to flow offers the simplest means of restricting the rates of bubbling and condensation. Since all of the gas passes into the lumen of the drain, there is appreciable windage at its upper end where the drops form. Too much windage causes the tip of each drop, when it grows large enough, to be pulled off into the drain, the greater portion of the drop remaining suspended upon the holder. A rapid series of droplets then passes down the drain while the main drop grows and shrinks slightly with the loss of each droplet. The result is a very small dropping fraction and poor extraction efficiency. This phenomenon can be avoided by keeping the gas flow rate below a certain critical value at which the gas flow begins to decrease the dropping fraction. I have found a rate of about 50 ml. per minute to provide a good compromise between efficient dripping action and efficient vapor transport. At 50 ml. per minute, the windage is not so great as to disturb the drop and yet the flow is great enough to provide a satisfactory rate of vapor transport.

The rate of vapor transport is considered satisfactory when the most volatile chemicals are transported in requisite quantities in no less than 10 or 15 seconds while the least volatile require no more than a minute and a half. If the volatility of a chemical and the rate of vapor transport are such that the requisite quantity is condensed upon the holder in less than 10 seconds, difficulties arise in timing the valve manipulations. Variations in vapor concentration in the reservoir **26,** variations in the depth of the liquid in the reservoirs or small variations of temperature will all have an appreciable effect upon the results with short times of transport of the chemical vapors. In manual operation, the holder can always be watched and any of these variations can be compensated by adjusting the duration of bubbling but if the bubbling becomes too brief, there may not be time to correct such errors. Thus, it is apparent that there is a relationship which exists amongst the gas flow or bubbling rate, reservoir temperature and vapor pressure for each chemical. For operation at a given reservoir temperature, chemicals of a suitable vapor pressure should be chosen.

I have found that adequate control can be effected by merely timing the duration of bubbling as indicated above, the nitrogen passing out the drain into the open atmosphere. This procedure raises the question of efficiency of the vapor transport which includes the efficiency of condensation of vapor upon the holder and the efficiency of evaporation in the reservoir. The limiting efficiency depends upon the temperature of the reservoir and the cool water used to chill the holder. It has been found satisfactory to use water at 20°C. while maintaining the reservoir and chamber at 50° to 60°C.

The theoretical ratio of the volume of liquid condensed to the volume of depleted carrier gas passing through the drain **12** is

$$MP_r(P_r-P_w)(P_1-P_2)/RDT_3(P_r-P_1)(P_r-P_2)$$

where M is the molecular weight of the vapor, P_r is the ambient atmospheric pressure, P_w is the vapor pressure of the water over which the gas is collected, T_3 is the temperature of the water, P_1 is the vapor pressure of the liquid at the reservoir temperature, P_2 is the vapor pressure of the liquid at the temperature of the sample holder **4**, D is the density of the liquid, and R is the gas constant. Measurements show that under practical conditions the efficiency of condensation of a solvent is about 60% of that predicted by the above expression, so that the total loss is about 50%. But since the consumption of chemicals amounts to only a few microliters per cycle, this loss is unimportant.

The more volatile chemicals suffer a strong cooling effect from evaporation during bubbling. Extensive transport of a solvent during extraction may cool the liquid in the reservoir well below the temperature of the housing. This is of little consequence for the extraction since the operation is normally carried on until a specified number of drops have been collected. However, after cessation of bubbling, the liquid in the reservoir begins to warm up again. Its vapor pressure then rises and some of it evaporates additionally into the vapor space of the reservoir. The vapor thus generated displaces the liquid into the backup bulb. This phenomenon makes backup bulbs indispensible. Besides, during initial warmup of the instrument, expansion of the inert gas as well as generation of vapor within the reservoirs **26** forces all the chemicals into the backup bulbs to varying degrees. In general it is preferable to employ chemicals having vapor pressures at the operating temperature no greater than one-half the ambient atmospheric pressure.

There is another circumstance which affects the flow of liquid between the reservoir and the backup bulb. If after delivering a chemical to the holder, valve **33** is closed before valve **21** is opened, gas flow stops during the interim. However, vapors present in the chamber continue to condense on the holder since the atmosphere in the chamber is never in equilibrium during flow. This condensation results in contraction of the gas within the chamber and suction of outside air through the drain **12**. Oxygen thus entering impinges directly upon the sample and can be very harmful. It is, therefore, vital to employ a valving sequence that will preclude entry of air. This is accomplished by never having both the valves **21** and **33** closed at the same time. In short, valve **21** must be open at all times except during actual bubbling of a chemical. The pressure in the apparatus will then never fall below the ambient atmospheric pressure. A suitable sequence for delivering, for example, two consecutive chemicals is as follows: valve **21** is open at the start; open valve **15**, open the first valve **33**, close valve **21**, bubble for the requisite time, open valve **21** whereupon bubbling stops owing to loss of the pressure drop across the reservoir, close the first valve **33**, open the second valve **33**, close the valve **21**, bubble for the requisite time, open valve **21**, close valve **33** and close valve **15**. In this sequence there are brief intervals when valves **21** and **33** are both open. At such times there is little pressure difference across the reservoir assembly. If the liquid stands higher in the reservoir than in the backup bulb, it will then flow backward under its head, driving gas up the tube **29** into the inlet manifold **25**. If the gas contains vapor of the chemical, it can diffuse into the other reservoirs, contaminating them. However, during normal operation the more volatile liquids stand appreciably higher in the reservoir only on cessation of bubbling, not at the start. Since the vapor in the backup bulb is swept out during bubbling, the reverse flow following its cessation carries little vapor into the manifold **25**. Nevertheless, these considerations make it advisable to close valve **33** promptly after opening valve **21**.

It is next appropriate to consider the function of the leaks **31**. It is important to prevent contamination of the chemicals by one another. When the present apparatus is not in operation, low pressure nitrogen supplied by the regulator **37** is maintained within the inlet manifold tube **25** and the backup bulbs **27**. Valve **21** is normally open at such time so that pressure is also maintained in the chamber. Valves **33** are, of course, closed. Under such static conditions, vapors rising in the backup bulbs would be free to diffuse through the tubes **29** into the inlet manifold **25** and thence into the backup bulbs of other reservoirs, being absorbed by the chemicals there present. However, the pressurized gas in the inlet manifold is at all times escaping through all of the leaks **31**. There is, therefore, a constant downward flow of gas through the tubes **29** and out through the side tubes **30**. The leaks are adjusted to provide flow rates of typically 3 to 7 ml. per minute. Since the bores of the tubes **29** are typically 1 mm., the linear flow rate of gas through tubes **29** and **30** is in the vicinity of 10 cm/sec. This suffices to sweep all vapors through the leaks.

Vapor can penetrate to the inlet manifold **25** only if the gas flow upward from the backup bulb into tube **29** exceeds the flow rate of the leak **31**. The apparatus is designed with a view to avoiding such excessive flow rate. Backward flow will occur during initial warmup of the instrument, immediately after bubbling while both valves **33** and **21** are open, and more slowly after valve **33** is closed after bubbling. In the first and last cases, the backward flow does not exceed the leak rate, which is adjusted to be larger for the larger reservoirs. In the second case, the backward flow may exceed the leak rate but, as pointed out above, the concentration of vapor in the flowing gas is small and the amount of vapor can be minimized by promptly closing valve **33**. Besides, the capillary parts of tubes **29** extend some distance above their junctions with the tubes **30**. This provides a small buffer space to receive a sudden surge of vapor that overshoots the leak; the continuing flow of gas sweeps the intruding vapor from this space. Moreover, the flow rate of the liquid from the reservoir into the backup bulb can be controlled by proper choice of bore and length of the tube **28**.

Flow of liquids in the reservoir assemblies and the generation of vapors in the backup bulbs are not the only causes of upward vapor flow through the tubes **29** with which the leaks **31** have to cope. Any manipulation of the various valves causes pressure changes within the manifold systems. If the surge is not so great as to drive vapors past the constricted parts of the tubes **29**, the leaks will carry them off. To keep the vapors out of the inlet manifold during such surges, the following factors are to be considered: the provision of an efficient pressure regulator that instantly adjusts to demand; low flow resistance in the manganous oxide container; and an operating pressure no greater than

needed for satisfactory bubbling, suitably not much exceeding 25 millibars.

There is one other point of leakage in the apparatus as described. This is the ground joint **2** at the reaction chamber. This joint should be dry, that is, no sealing grease or the like should be used since it would absorb vapors from the reaction mixture and act as an undesirable sink and source. Thus, the joint will leak since the chamber is always under pressure except when the drain or vent is open and gas is flowing through them. When the joint is dry, pressure of gas will tend to lift the male member of the chamber so that it floats within the female member permitting rapid leakage. To prevent this, the male member can be weighted or otherwise tied down. Some leakage, however, is beneficial in that it tends to sweep out vapors trapped within the joint and adsorbed upon its surfaces. On the other hand, the leakage should not be so great that it carries off an appreciable quantity of the vapors present in the chamber during coupling, permitting evaporation of the coupling solution. A leak rate not much exceeding 0.1 ml. per minute at 25 millibars may be considered acceptable.

It is known that oxygen can enter an oxygen-free apparatus by diffusing inward through existing leaks despite an active outward flow of gas through the leaks. However, the leaks of the present invention, being long and narrow, impede upstream diffusion of oxygen. Monitoring of the green manganous oxide at the downstream end of the container **38** has shown that the average concentration of oxygen within the apparatus cannot much exceed 0.01 ppm. This is three to four orders of magnitude below the concentrations prevailing in commercial sequencers.

The following four factors pertaining to the reaction chamber affect the vapor transport. (1) The components of the coupling reagent are condensed serially. Each component after the first condenses not upon the clean holder or dry sample but upon a liquid that differs from it and that can dissolve it. The partial pressure of each component is less in the solution than it was when pure. This effect increases the efficiency of condensation and slightly raises the vapor transport. (2) Each component but the last is exposed to a continuing flow of inert gas carrying none of its vapor. A portion of it must, therefore, evaporate after it has been deposited. This loss is lower, the cooler the holder, and the greater the depression of the vapor pressure by the components arriving at a later time. The loss can be minimized by depositing the components of the coupling reagent in the order of diminishing bubbling times. (3) After the last component has been deposited upon the holder, the drain valve **15** is closed, leaving the atmosphere in the chamber static. The holder is then warmed to the coupling temperature, whereupon the vapor pressures of all components increase greatly and they begin to evaporate. The warming and the evaporation cause a slight backward expansion of gas from the chamber through the outlet manifold **20** and valve **21** which, as has been mentioned, is open at all times except during bubbling. The evaporation quickly comes to a stop as all the gas space immediately accessible to the holder becomes saturated with the vapors of the coupling mixture. (4) There is a slight continuing evaporation during coupling as gas slowly moves through the chamber and out through the leak **2**.

These four effects are hard to predict. The last two, with proper chamber design as in FIG. **4**, are much smaller than the second. The second factor is believed not to exceed 10% or at most 15% of the amount condensed. However, these uncertainties, as well as the effect of depletion of the chemicals in the reservoirs upon the degree of saturation and flow rate of the bubbles, are evidently unimportant in the present invention, since obvious variations in reagent volumes have not much affected the efficiency of degradation. In prior art sequencers, on the other hand, such variations can be disastrous.

The valves **33** will next be described. For these valves and for the gas valve **21**, "Rotaflo" valves manufactured by Quickfit, Inc. of Fairfield, New Jersey have been found satisfactory. As shown in FIG. **5**, the valve has a borosilicate glass body **51** with a straight tubulation **52**, which shall arbitrarily be called the inlet, and a side tubulation **53** at right angles to the body, which shall arbitrarily be called the outlet. The key or core **54** is a cylindrical plug of polytetrafluoroethylene (PTFE). The lower end **55** of the core **54** facing the inlet **52** seats against a shoulder **56** to effect closure. The upper portion of the core **54** contains packing which can be compressed by a screw **58** to make the upper wall **59** of the core press against the glass body **51**. The upper end of the core **54** fits a handle **61** which is attached to the core by means of a screw **62**. The screw **62** has an axial hole **63** through which a slender screwdriver can be inserted to adjust the packing screw **58**. The handle **61** is a nut fitting on screw threads **64** on the outside of the body **51**.

The PTFE is permeable to various vapors. Although the valves do not leak when closed, the vapors in the reservoirs slowly diffuse through the PTFE cores and enter the outlet manifold **20**, ultimately reaching the chamber **1**. The permeation is harmless in the case of most of the chemicals, but it may cause trouble in the case of the buffering amine and the cleaving acid. If it enables the acid and amine to come together at any time, "dew" will form.

Because of the permeability of PTFE to the acid, there is provided a special valving system for the acid. This, together with other features of the valve and manifold design, is shown schematically in FIG. **6**, which represents the gas flow scheme more abstractly than FIG. **2**. Nitrogen enters through tube **22** and passes to the gas valve **21** and the various reservoir assemblies. All leaks and outlet valves in the apparatus are represented by outwardly directed arrows. The backup bulb inlet tubes **29** and the leaks **31** are shown. For minimal contamination, the amine is placed in the penultimate reservoir **64**a (with its backup bulb **65**) and the acid in the last (downstream) reservoir **66** (with its backup bulb **67**), nearest reaction chamber **1**. The drain valve **15** and vent valve **46** are shown attached to the chamber, and the leakage through the joint **2** is represented by the arrow **2**. The reservoir assemblies **26**. **27** other than those of the acid and amine, with their valves **33**, typically five in number, are located between the amine assembly **64**a, **65** and the gas valve **21**. The arrows within the circles representing the valves point from the "inlet" **52** to the "outlet" **53** as shown in FIG. **5**. The chemical valves from the first to the antepenultimate one, all marked **33** in FIG. **6**, are oriented in the "normal" direction, the arrows pointing in the direction of flow. In these valves the packing is on the downstream

side. Since each of these valves is mounted vertically in the position shown in FIG. 5, connected by a straight, vertical tube 32 (a continuation of tube 52) to the reservoir 26 directly below, removal of the key provides direct, straight access, as by means of a pipette, to the reservoir. This arrangement is feasible with the reservoirs other than those of the amine and acid because permeation through and absorption by the PTFE are unimportant and it makes little difference which way the valve is directed.

The amine valve 68, analogous to those marked 33, is mounted in reverse direction. The purpose is to keep the core on the upstream side where it is always exposed to the amine vapor and cannot act as a sink-and-source with respect to the space on the downstream side comprising the outlet manifold tube 20 and chamber 1. If valve 68 were mounted in the normal direction, the core would absorb amine vapor when the valve was open and release it slowly afterward. In the reverse orientation, the outlet 53, now actually the inlet, is connected by a smoothly curved right angle bend to the vertical reservoir outlet tube 32. The inlet 52, now actually the outlet, is joined very close to the seat 56 to the outlet manifold tube 20 at a joint 34. Removal of the key of valve 68 does not provide straight-through access to the reservoir 64*a*. To fill the reservoir, a funnel or pipette extended by a slender, flexible PTFE tube is threaded through the open body 51 and the outlet 53 into the tube 32 and the reservoir 64*a*.

The acid reservoir assembly 66, 67 is joined to the outlet manifold and vapor tube 19 through two valves 69 and 70. These valves are like the others and are essentially as shown in FIG. 5. The upstream valve 69 is oriented in the normal direction and is joined directly to the acid reservoir 66 by a vertical, straight tube 32 in the same way as all the other chemical valves except that of the amine. Removal of the key of valve 69 thus affords direct, easy access to the reservoir. The downstream valve 70 is oriented in the reverse direction and is connected at a compact joint 34 (very close to the valve seat) to the vapor tube 19 in the same way as the amine valve 68. The valves 69 and 70 differ from the others in having a fine groove scored longitudinally in the surface of the core of each. This groove causes each valve packing to leak, suitably at a rate of about 4 ml. per minute under a pressure of 25 millibars. The two valves are connected at their outlets, the connecting tube being a short glass capillary 71. The leaks in the cores are symbolized by the arrows 72, 73. The connecting tube 71 is connected at a T-joint 74 in its center to a tube 75 that is an extension of the inlet manifold 25 and joins it at the connection 50 at which the tube 29 leads off to the acid backup bulb 67. The leaks 72 and 73 are in parallel with the leaks 31; inert gas flows continually through all of them under the pressure prevailing in the inlet manifold. The space between the seats of the two valves 69 and 70, comprising the body 51 of each valve and the connecting tube 71, is thereby continually purged by an outward flow of inert gas, carrying off any vapor that is trapped between the valves after delivery of acid. In this way, the upstream side of valve 70 is kept largely free from acid vapor so that very little acid can permeate this valve when it is closed. Moreover, its "reversed" orientation has the same advantage as it does with the amine valve 68 in keeping the bulk of the valve core on the upstream side.

In the arrangement as described up to this point, if valves 15, 69 and 70 were to be opened and valve 21 closed for the purpose of delivering acid vapor to the holder, no bubbling would take place. Since valve 69 together with the connecting tube 71 and junction 74 offers little flow resistance, the pressure on the two sides of reservoir 66 would be nearly equilibrated through the tube 75. The gas would merely flow through inlet manifold 25, extension tube 75, valve 70, and vapor tube 19 to the chamber, bypassing the acid supply. To create a pressure drop across the acid sufficient to drive bubbles through it for delivery of vapor, a narrow constriction 76 is created in tube 75 by heating it with a torch to shrink the bore. A constriction such that nitrogen will flow through it at a rate not exceeding 50 ml. per minute under a pressure drop of 25 millibars is probably satisfactory. When constriction 76 has been installed, there will be enough pressure drop across reservoir 66 to start bubbling when valves 15, 69 and 70 are open and valve 21 is closed. On the other hand, the constriction 76 does not impede the flushing action of the leaks 72 and 73 when valves 69 and 70 are closed, since these leaks offer much more flow resistance than the constriction does. In case the constriction is not resistive enough to ensure commencement of bubbling when valve 15 is open, momentary opening of the vent valve 46 will augment the pressure drop across reservoir 66 and start the bubbling action; valve 46 can then be closed without halting the flow of bubbles.

As shown in FIGS. 1 and 6, there is a short connecting tube 77 between each valve 33 and the outlet manifold 20. Since the volume of the tube 77 is small (about 0.15 ml.), the flow of nitrogen through the outlet manifold 20 flushes most of the vapor out of the tube 77. However, in the case of amine and acid reagents, this flushing must be more efficient to prevent contamination by these reagents.

FIG. 7 illustrates a preferred connection between valve 33 and outlet manifold 20 which permits more efficient flushing of the tube 77. The upstream portion of the outlet manifold 20 (identified in FIG. 7 by the reference numeral 78) approaches the valve 68 or 70 in an upwardly directed arc 79, whereby the flow of nitrogen is directed against the seat 56 of the valve with the result that residual vapor is efficiently flushed away. The downstream portion of the outlet manifold 20 (identified in FIG. 7 as reference numeral 80) is connected as close as possible to the seat 56 to facilitate removal of the vapor.

The apparatus requires a drain 12 and some embodiments have an additional vent 44. Since these must be closed during coupling and cleavage, the design of valves to close them is important. They should be leak-tight and virtually noncontaminating. I have found rubber pads like those marked 16 and 46 to provide satisfactory closure. The tip is tapered to a thin-walled orifice, the bore remaining more or less constant. The circular edge of the orifice, if it does not lie in one plane, is ground down until it does, and is then fire-polished. The resulting smooth, circular orifice is lightly pressed against by the rubber pad 16, which is slightly indented by it, creating a leak-tight seal. Although rubber readily absorbs the vapors of the chemicals and can, therefore, act as a sink-and-source, its exposed area within the lumen of the orifice 14 is so small and the length of the drain 12 (or the vent 44) is so long relative to its cross

section, that the contaminating action of the rubber valve pad is negligible. Any suitable means may be used to apply the rubber pad **16** or **46** to the drain **12** or the vent **44.**

At this point the procedure for sequencing a sample of protein or peptide will be described. After the glass assembly has been thoroughly cleaned, reagents and solvents are charged to their respective reservoirs, preferably in the following sequence: butyl chloride, ethyl acetate, n-propanol, water, phenyl isothiocyanate, dimethyl benzyl amine, heptafluorobutyric acid. The sequence is from upstream to downstream (clockwise in FIG. 2). Each chemical is charged by adjusting regulator **37** to give a low pressure of nitrogen (about 15 millibars), removing key **54** after loosening the packing screw **58** of the valve **33,** and then delivering the chemical to the reservoir through a pipette or funnel. Due to the nitrogen pressure, each chemical bubbles until the valve **33** is reassembled. However, since bubbling purges the chemical of oxygen, it is desirable to continue bubbling for about 5 minutes by opening valve **33** and the drain **12** and vent **44** while keeping valve **21** closed. Before the next chemical is charged, valve **33** is closed and valve **21** is opened to flush the outlet manifold **20** and the chamber **1.** All valves are then closed.

After the sample holder **4** has been thoroughly cleaned, the sample of protein or peptide is applied with a syringe to the tip **10** of the sample holder as a solution or suspension in a suitable medium, such as water in the case of insulin or trifluoroacetic acid in the case of myoglobin. To avoid dripping, preferably not more than about 10 microliters of sample is applied. The sample preferably contains from about 1 to 10 nanomoles of the peptide or protein. The sample is dried by circulating warm water through the tube **5** and passing nitrogen through the chamber **1.**

The following program, offered as an example that may be suitable for sequencing peptides, shows a detailed procedure for completion of a single cycle of degradation. It will be understood that every detail is under the control of the operator and can be varied at will.

Housing, 65°C. Water, 60° and 20°. Pressure, 25 millibars.

Function	Chemical	Gas	Water	Drain	Vent	Time
(Standby)	—	On	Hot	Shut	Shut	—
Deliver	PITC	Off	Cool	Open	"	80 sec
Flush	—	On	"	"	"	few sec
Deliver	Water	Off	"	"	"	37 sec
Flush	—	On	"	"	"	few sec
Deliver	Propanol	Off	"	"	"	13 sec
Flush	—	On	"	"	"	few sec
Deliver	Amine	Off	"	"	"	13 sec
Flush	—	On	"	"	"	few sec
Couple	—	On	Hot	Shut	"	12 min
Dry	—	On	"	Open	"	2 min
Purge	—	On	"	"	Open	15 sec
Deliver	Acid	Off	Cool	"	Shut	35 sec
Flush	—	On	Cool	Open	Shut	few sec
Cleave	—	"	Hot	Shut	"	2 min
Dry	—	"	"	Open	"	2 min
Purge	—	"	"	"	Open	15 sec
Extract	BuCl	Off	Cool	Fraction	Shut	4 drops
Dry	—	On	Hot	Open	Shut	1 min
Purge	—	"	"	"	Open	15 sec
(Standby)	—	"	"	Shut	Shut	—

The quantities of the chemicals deposited in the coupling mixture are probably about as follows: phenyl isothiocyanate, **0.6** μl; water, 3.5 μl; propanol, 5 μl; dimethyl benzyl amine, 1 μl. In the table, "Gas" refers to valve 21. The details of manipulation of valve 21 and the valves 33 during delivery of a chemical have been given above. The "flush" function following each delivery refers to the brief interval after the appropriate valve 33 has been closed, valve 21 being of course then open, and before the next function (such as delivery of another chemical) begins. During this interval, the nitrogen sweeps vapors out of the outlet manifold. The drain 12 should be shut before the water is changed from cool to hot to avoid premature evaporation. The amine in the above program is dimethyl benzyl amine; the acid is heptafluorobutyric acid. The work "Fraction" under "Drain" at the extraction step signifies that the fraction container is placed under the drain and removed when extraction is complete. The window cover should be left in place except when the bubbling action, the condition of the chamber, or the operation of extraction is to be inspected or watched. The housing temperature must be checked and adjusted as needed.

Identification of the amino acid residues collected in each fraction is preferably carried out concomitantly with the sequencing operation. Identification may be carried out in accordance with the following procedure. To the dried extract in fraction container **18,** add 0.04 ml of 1-N hydrochloric acid, swirl, purge for about 20 seconds with nitrogen, stopper the container, heat at 80°C. for about 5 minutes, cool quickly, add 0.04 ml of ethyl acetate, swirl, centrifuge briefly, decant the extract into a glass-stoppered tube, repeat the extraction, evaporate the combined extract, mix with 6 microliters of ethyl acetate, and withdraw 2 microliters for analysis by gas chromatography. The identification is conveniently carried out while the succeeding cycle is being run.

A sequencing run was continued through 18 cycles of a 5-nanomole sample of myoglobin. It was coupled 15 minutes at 50°, washed with three drops of benzene, cleaved 3 minutes at 50°, and extracted with four drops of ethyl acetate. The cycle time was as short as 38 minutes. FIGS. **8A** and **8B** present copies of gas chromatograms of 40% aliquots of the 15th fraction (alanine) and the 17th fraction (valine). These chromatograms are remarkably clean, revealing only the phenylthiohydantoins of the amino acids in the region of interest. The identifications are unequivocal. The valine and leucine peaks in No. 15 and the alanine and leucine peaks in No. 17 are due to a combination of asynchrony (overlap) and nonspecific cleavage. The level of nonspecific cleavage is no greater in this run than that commonly encountered in commercially available sequencers. The average epitopic repetitive yield was 90% and the average pantopic repetitive yield was 93–94%; but beyond the eighth cycle the repetitive yield seems to have risen to 97%, a value rarely attained in any kind of sequencing work. The asynchrony, however, averaged 2% per cycle, which is higher than it should be and is due probably to the short coupling time.

Another sequencing run was continued through 18 cycles of a **5**-nanomole sample of insulin A. It was coupled 10 min. at 60°, not washed, cleaved 1 min., and extracted with four drops of butyl chloride. The cycle time was as short as 24 minutes. FIGS. **9A, 9B** and **9C** presents gas chromatograms of 40% aliquots of the seventh fraction (cysteine), the eighth fraction (threonine), and the 10th fraction (isoleucine), showing clear

identifications and excellent yields. The epitopic repetitive yield was 87% and the pantopic repetitive yield was 93%, which is exceptionally high for a short peptide. The asynchrony amounted to about 3% per cycle, but such asynchrony with insulin is not unusual even with the highly perfected programs now available for commercial sequencers.

Thus, there has been provided a simple, easy to operate, inexpensive apparatus capable of rapidly and efficiently sequencing small quantities of peptides and proteins with correspondingly small quantities of chemicals.

What is claimed is:

1. A sequencer comprising a reaction chamber having inlet and outlet openings, a sample holder extending downwardly into said chamber and serving to retain a sample on a lower surface thereof, means for selectively heating and cooling said sample holder, and means for selectively introducing chemical vapors and inert gas into said reaction chamber whereby said chemical vapors can be condensed on the sample holder when said sample holder is cooled and the liquid on said sample holder can be evaporated by the inert gas when said sample holder is heated.

2. A sequencer as in claim **1** wherein said means for selectively introducing chemical vapors comprises a plurality of reservoirs adapted to retain the chemicals in fluid form, a source of inert gas connected to each of said reservoirs for bubbling said inert gas through said chemicals and valve means for selectively connecting the inlet of said chamber to a selected reservoir whereby the inert gas flows through the chemical in said selected reservoir to form and transport a chemical vapor of the selected chemical into the reaction chamber.

3. A sequencer as in claim **1** wherein said sample holder is removable from the chamber to permit introduction of a sample on a lower surface of the sample holder.

4. A sequencer as in claim **1** wherein said inlet is near the bottom of the chamber.

5. A sequencer as in claim **1** wherein said sample holder comprises a closed hollow finger-shaped member and wherein said means for selectively heating and cooling said finger includes means for circulating liquid therethrough.

6. A sequencer as in claim **5** in which a first tube extends into and is spaced from the holder with its lower end secured to the lower end of the holder and a second tube extends downwardly within the first tube to a point spaced from the lower end of the holder whereby liquid can circulate in the annular space between the first and second tube and through the second tube.

7. A sequencer as in claim **1** wherein said outlet opening comprises a drain disposed directly beneath said surface of said sample holder retaining said sample, said drain serving to permit the exhaust of inert gas and vapors introduced into said chamber and the removal from said chamber of liquid droplets falling from said lower surface of said sample holder.

8. A sequencer as in claim **7** including valve means for selectively opening and closing said drain.

9. A sequencer as in claim **7** wherein said inlet is near the bottom of the chamber and said drain extends upwardly into the reaction chamber above said inlet.

10. A sequencer as in claim **7** including a vent communicating with said chamber and valve means for selectively opening and closing said vent whereby to provide means for purging said chamber.

11. A sequencer as in claim **10** wherein said vent offers substantially less resistance to flow of gas than said drain.

12. A sequencer as in claim **7** wherein the drain comprises a tube having a flat, horizontal upper end with a rounded interior edge, the tube extending upwardly into the reaction chamber such that the gap between the top of the tube and the tip of the holder is that at which liquid will drip from the holder to the tube leaving an amount of liquid clinging to the holder which is a minimal fraction of the volume of the drop.

13. A sequencer as in claim **12** wherein the gap is about 1.4 mm.

14. A sequencer as in claim **12** wherein the tip of the holder has a rough surface.

15. A sequencer as in claim **12** wherein the bore of the tube is such that the flow rate of inert gas through the tube is less than that at which the fraction of liquid clinging to the holder after each drop begins to increase.

16. A sequencer as in claim **15** wherein the bore is such that with a pressure of inert gas suitable for operating the sequencer the flow rate is about 50 ml. per minute.

17. A sequencer comprising a reaction chamber having inlet and outlet openings, a sample holder removably mounted at the top of said chamber and extending downwardly into said chamber, means for selectively heating and cooling said sample holder, an outlet manifold connected to said inlet opening, a supply manifold adapted to be connected to a source of inert gas, a plurality of reservoirs for storing chemicals used in sequencing a sample connected between the supply manifold and the outlet manifold, and valve means connected in series with each of said reservoirs such that the supply manifold can be selectively connected to the outlet manifold through a selected reservoir thereby permitting the inert gas to come into contact with the chemical in the selected reservoir to convey the vapor of the chemical to the outlet manifold for introduction into the reaction chamber.

18. A sequencer as in claim **17** wherein each reservoir comprises a first container connected to the supply manifold and a second container connected through said valve means to said outlet manifold, the two containers being joined at their lower ends by a connecting tube, whereby when said valve means is opened the inert gas forces the chemical in the reservoir into the second container and bubbles through it and when the valve means is closed the chemical is free to flow back into the first container.

19. A sequencer as in claim **17** including means for preventing the vapor of chemicals in any reservoir from entering another reservoir.

20. A sequencer as in claim **19** wherein said means comprises a leak permitting the flow of inert gas to the atmosphere from the connection between the supply manifold and each reservoir.

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