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A Protein Sequenator

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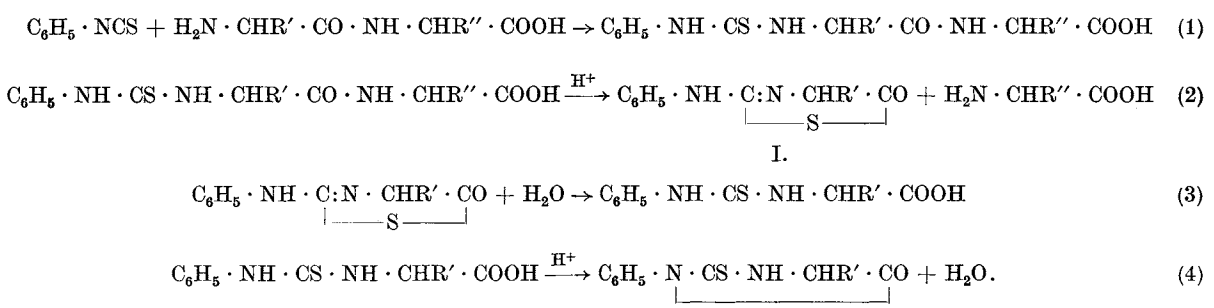
The protein sequenator is an instrument for the automatic determination of amino acid sequences in proteins and peptides. It operates on the principle of the phenylisothiocyanate degradation scheme. The automated process embraces the formation of the phenylthiocarbamyl derivative of the protein and the splitting off of the N-terminal amino acid as thiazolinone. The degradation proceeds at a rate of 15.4 cycles in 24 hours and with a yield in the individual cycle in excess of 98%. The material requirements are approximately 0.25 μmoles of protein. The thiazolinones are converted to the corresponding phenylthiohydantoins in a separate operation, and the latter identified by thin layer chromatography. The process has been applied to the whole molecule of apomyoglobin from the humpback whale, and it has been possible to establish the sequence of the first 60 amino acids from the N-terminal end.

Present techniques for the elucidation of protein primary structure are highly time- and labor-consuming, and quite inadequate when seen in relation to the vast amount of work waiting to be done. This contribution describes an attempt to create a more favorable situation.

The scheme is based on the phenylisothiocyanate reaction for determining amino acid sequences. In principle this procedure should allow the degradation of any sequence regardless of length, but in practice only short degradations have been feasible. The termination of a degradation is usually caused by diminishing yields, but also overlapping between consecutive steps, and nonspecific cleavage along the peptide chain tend gradually to obscure the results. For larger structures, like proteins, there is the added problem of poor solubility in the media of the degradation. However, these limitations may be made less severe by proper measures. For this an understanding of the reaction mechanism is essential [1,2]:

These reactions will be referred to as coupling (1), cleavage (2), and conversion (3, 4). The 2-anilino-5-thiazolinone derivatives (I) are too unstable to make them suitable for identification purposes, and are therefore converted to the isomeric 3-phenyl-2-thiohydantoins (PTHs). Several features of the mechanism have a bearing on the degradation procedure. First, reaction (2) is fast whereas reaction (4) is slow. It is therefore advantageous to separate the thiazolinone from the shortened peptide, and convert it separately to the corresponding phenylthiohydantoin, thus minimizing the exposure of the peptide to strong acid. Second, reaction (2) proceeds readily under anhydrous conditions, and it is therefore possible to eliminate any hydrolytic action by the acid on the peptide. Finally, the phenylthiocarbamyl group has been found to be easily desulfurized by oxidation, even under the influence of oxygen dissolved in the medium [3]. This brings the degradation to a halt since obviously a thiazolinone can no longer be



I.

Non-standard abbreviations and trademarks. 3-Phenyl-2-thiohydantoin, PTH; polytetrafluoroethylene, PTFE; *N,N,N',N'*-tetrakis-(2-hydroxypropyl)-ethylenediamine, Quadrol®.

formed. This side reaction may be eliminated simply by carrying out the procedure in an inert gas.

The various amino acids do not differ greatly in reactivity during the degradation process, and it is feasible to find one set of reaction conditions covering all cases. The degradation of a peptide may then be reduced to a repetition of a standard set of conditions. Such a degradation cycle would lend itself to programming. The theme of this contribution is the design of an instrument based on this principle.

We propose the term 'sequenator' for an instrument which determines the sequence of an ordered linear polymer by repeating a chemical process.

DESIGN

The general design of the sequenator is shown in Fig. 1. The reaction vessel is a cylindrical glass cup (A) mounted on the shaft of an electrical motor (B). The cup spins continuously, and solutions and solvents entering the cup are therefore spread as thin films on the walls of the cup. Reagents and solvents enter through the feed line (R) at the bottom of the cup. Extracting solvents climb to the groove where they are scooped off, and leave through the effluent line (S). The cup is enclosed in a bell jar (Q), and the system can be evacuated by means of a vacuum pump (P). The system is also thermostated.

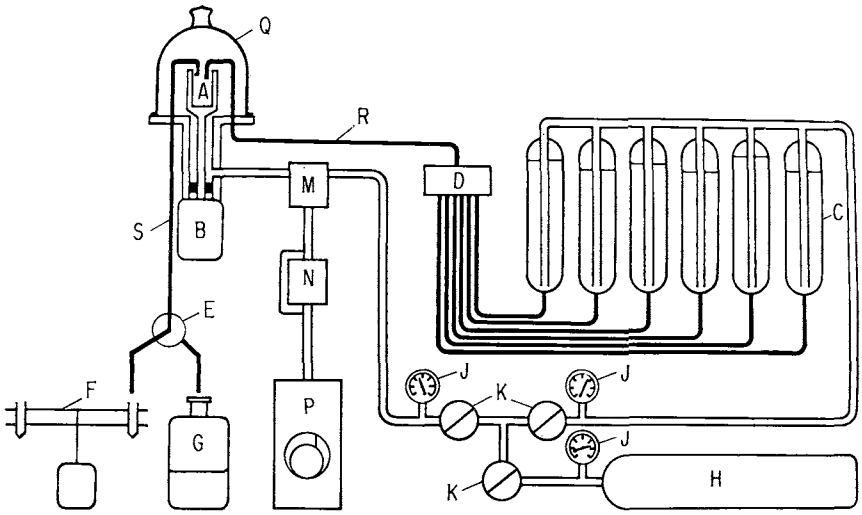


Fig. 1. *Diagram of sequenator.* *A*, spinning cup; *B*, electric motor; *C*, reagent (solvent) reservoir; *D*, valve assembly; *E*, outlet stopcock assembly; *F*, fraction collector; *G*, waste container; *H*, nitrogen cylinder; *J*, pressure gauges; *K*, pressure regulators; *M*, 3-way valve; *N*, 2-way valve with bypass; *P*, rotary vacuum pump; *Q*, bell jar; *R*, feed line; *S*, effluent line. Gas lines are doubly contoured, and liquid lines are filled

The design problem may be simplified by leaving the conversion reaction out of the considerations. This can be done without great sacrifice since this reaction can conveniently be carried out on a large number of samples simultaneously, and is therefore of no importance for the speed of the analysis.

The coupling and the cleavage procedure both call for a number of mechanically diverse operations, *e.g.* extractions, centrifugations, and dryings. In order to make the design simple it is desirable to have one mechanical operation accommodating all these processes. This has been achieved by spreading the solutions in thin films inside a rotating cylindrical cup. The spinning film is ideally suited for extraction by another film of an immiscible solvent sliding over its surface, for centrifugation and also for drying under reduced pressure, because of the large surface and the stabilizing centrifugal force.

A preliminary account of this work has appeared [4].

Reagents and solvents are stored in reservoirs (C), and are admitted to the cup through an assembly of valves (D). The reservoirs are under a constant low pressure of nitrogen supplied by a nitrogen cylinder (H) and pressure regulators (K). The contents of the bell jar are likewise held at a fixed although lower pressure of nitrogen through a similar arrangement. The pressure differerential between the reservoirs and the bell jar is constant, and the volume of reagent or solvent admitted to the cup is therefore determined by the time a valve is kept open.

The effluent line leads *via* a 3-way stopcock (E) either to a fraction collector (F) or to a waste container (G).

The valves in the assembly (D) and the gas valves (M and N) are operated by solenoids, and the 3-way outlet stopcock (E) and the fraction collector (F) by electric motors. All these functions are governed by an electronic programming unit.

The motors driving the cup and the vacuum pump run continuously.

The corrosive nature of several of the reagents used in the degradation severely limits the range of materials that can be used in the construction. Only borosilicate glass, PTFE, and gold are used in direct contact with reagents. Where the contact is solely with vapors, stainless steel (SAE 30321) is also employed.

Since highly inflammable solvents are used care has been taken to eliminate the explosion hazard by excluding sparking electric contacts. Where this is not feasible contacts are hermetically enclosed.

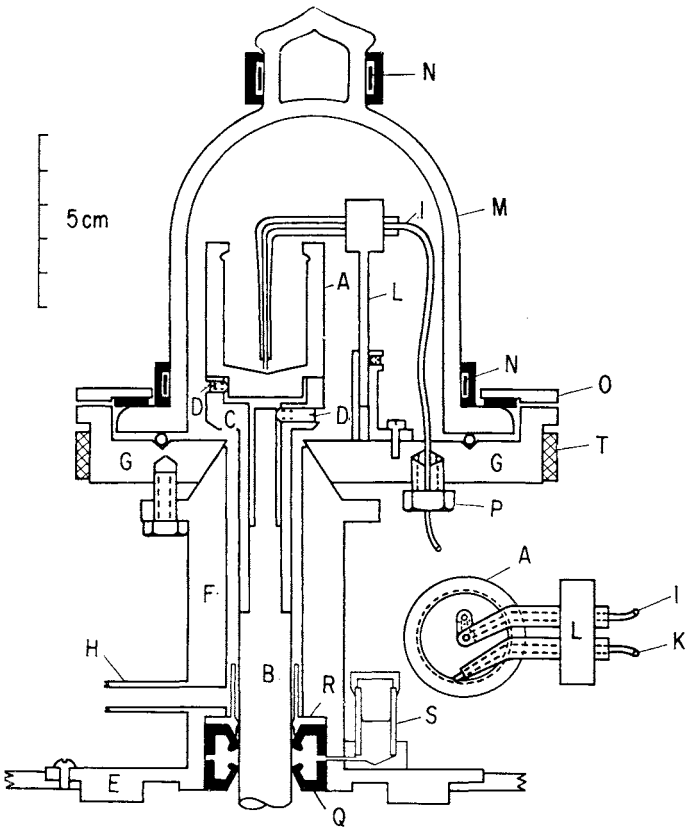


Fig. 2. *Reaction chamber.* *A*, glass cup; *B*, motor shaft extension; *C*, cup support; *D*, grub screws; *E*, motor support; *F*, column; *G*, base plate; *H*, side tube; *I*, feed line; *K*, effluent line; *L*, adjustable stand; *M*, bell jar; *N*, electrodes; *O*, rubber padded ring; *P*, sealing bolt; *Q*, oil seal; *R*, PTFE sleeve; *S*, oil reservoir; *T*, band heater. A top view of the cup, the feed line, and the effluent line is shown in the lower right hand corner

Rotor Assembly

It is essential for a correct operation of the cup that the inside cylindrical surface should run true. If this is not the case, disturbances in the liquid will result in mixing of the top and bottom part of the contents with diminished efficiency of the extraction as a consequence. The tolerance requirements adhered to are, that no part of the inside wall should vary more than 10 μ in distance from the rotational axis. These requirements make considerable demands on precision in manufacturing the cup, as well as on its alignment and on motor bearings (Fig. 2).

Cup. The cup is made of Pyrex glass. The inside diameter is 26 mm, and the height of the inside cylindrical wall is 31 mm as measured from the bottom to the groove. The groove has a depth of 1 mm relative to the inside wall. The opening of the cup is slightly narrower, 24 mm in diameter, to prevent splashing during the scooping operation. The inside bottom of the cup is dished to assist withdrawal of the contents when the cup is stationary.

Motor. The requirements are a suitable and constant speed and freedom from sparking. A 3-phase, 4-pole induction motor with the ratings 1,425 rev./min and $^1/_2$ H.P. (G.M.F. Electric Motors Pty. Ltd., Arncliffe, N.S.W., Australia) is used. The power rating is far in excess of demands but the larger physical dimensions have the advantage of making the hermetic sealing of the motor shaft easier. The original ball bearings are replaced by bearings with closer tolerances (SKF Ball Bearing Co. Pty. Ltd., Melbourne, Australia, types RLS 5C2 and RLS 6C2). The top end of the motor shaft is extended by a stainless steel shaft heatshrunk on to the original shaft. The shaft extension has its diameter reduced in two steps to receive the cup support and the aligning screws respectively.

Cup Support. This is made of stainless steel. It slides freely on the extended motor shaft when not locked in position. Three PTFE-tipped grub screws hold the cup. Three other screws impinge on the top section of the extended motor shaft, and serve to align the cup within the prescribed tolerances. The alignment is made with the help of a dial gauge. A coarse alignment is first made of the outside surface of the cup support. The fine alignment is made with the feeler arm of the dial gauge resting on the inside cylindrical surface of the cup and with manual rotation of the shaft. Both the top and the bottom part of the surface conform to the tolerance limits of 10 μ.

Motor Support, Column, and Base Plate

These are the supporting structures, and are made of stainless steel. The motor support and the column are joined into one piece by silver brazing. The column and the base plate are connected in a conical joint firmly held together by three bolts. A piece of unsintered PTFE tape between the tapered surfaces ensures the vacuum seal. The column has a side tube connecting it to the 3-way vacuum-pressure valve (Fig. 1, M). The motor is firmly bolted, through its top end plate, to the motor support, and the latter is fixed to the supporting framework.

Feed Line and Effluent Line

These are PTFE tubes with inner diameters 0.5 mm and 0.9 mm respectively. They are guided into their correct positions in the cup by capillary glass tubes supported by an adjustable stand. The feed line, which is approx. 30 cm in length, terminates at the bottom of the cup somewhat off center, and with just so much clearance from the bottom that liquid flow is not obstructed. The effluent line enters the cup as nearly tangentially as possible with the tip located in the groove and facing the rotational direction of the cup. There is a small clearance all around between the tip and the groove, and the walls of the tip are thinned to reduce turbulence. The scooping is assisted by the momentum of the rotating liquid and by the higher gas pressure in the bell jar.

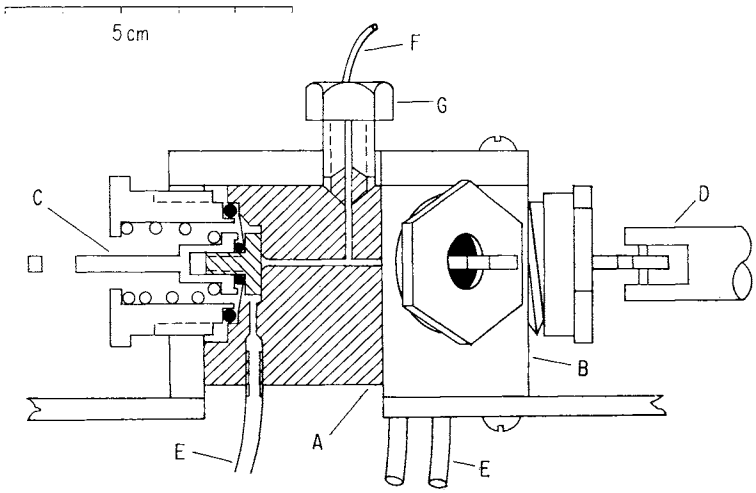


Fig. 3. *Valve assembly.* *A*, PTFE block; *B*, stainless steel casing; *C*, S.S. shaft; *D*, solenoid core; *E*, PTFE tubes from reservoirs; *F*, feed line; *G*, sealing bolt

Bell Jar

The jar is made from a Pyrex flat flange joint (Quickfit and Quartz Ltd., Stone, Staffordshire, England, type FG 75). The volume of the bell jar is kept as small as possible in order to reduce gas space. To allow heating the outer surface has an electrically conductive coating [5], and bandshaped silver electrodes surround the bell jar at top and bottom.

Vacuum Seals

The seal between the bell jar and the base plate is provided by a PTFE O-ring. A rubber-padded stainless steel ring on top of the glass flange is bolted down to the base plate, and compresses the O-ring.

The PTFE tubes passing through the base plate are sealed by PTFE ferrules compressed by bolts.

The seal around the motor shaft is designed to ensure against leaks from the outside during vacuum stages, as well as against leaks in the opposite direction when the pressure in the bell jar is above atmospheric. It should also withstand the action of organic vapors. Two identical oil seals (Super Seals Pty. Ltd., Melbourne, Australia, type 13720N10-ABO1) facing each other and a PTFE sleeve are forced into a recess at the bottom of the column. The lips of the seals close on the shaft. The corresponding area of the shaft is polished to a mirror finish. The space between the seals and the shaft is filled with vacuum oil. The PTFE sleeve serves to reduce the contact of the synthetic rubber oil seals with organic liquids and vapors. However, the seals tend to harden with time, and should then be replaced.

Heating and Thermostating

A band heater (300 watts) is clamped around the circumference of the base plate. The base plate has a thermistor heat-sensing element, and is thermostated. The bell jar has an electrically conductive coating as already described.

The temperature of the system is fixed during actual operation by first letting the base plate reach the temperature set by the thermostat. The voltage across the electrodes of the bell jar is then increased to a point where liquid no longer condenses on the walls. It has been found that a temperature setting of 50° requires approximately 35 volts (A.C.) across the electrodes.

Valve Assembly

This allows the measured admittance of reagents and solvents to the reaction cup. The design (Fig. 3) should fulfil several requirements. First, an accurate dispensing by timing requires, particularly for smaller volumes, that opening and closing of the valves should be instantaneous. Second, the dead volume

between the valves and the cup should be mininal. Finally, the valves should seal off volatile solvents effectively enough for the maintenance of an adequate vacuum in the bell jar during the vacuum stages.

The assembly consists of a cylindrical block of PTFE enclosed in a hexagonal stainless steel casing and six valves projecting radially at angles of 60°. The seats of the valves are formed by the PTFE block. The moving parts are cylindrical pieces of gold. The sealing surfaces are flat and polished. The

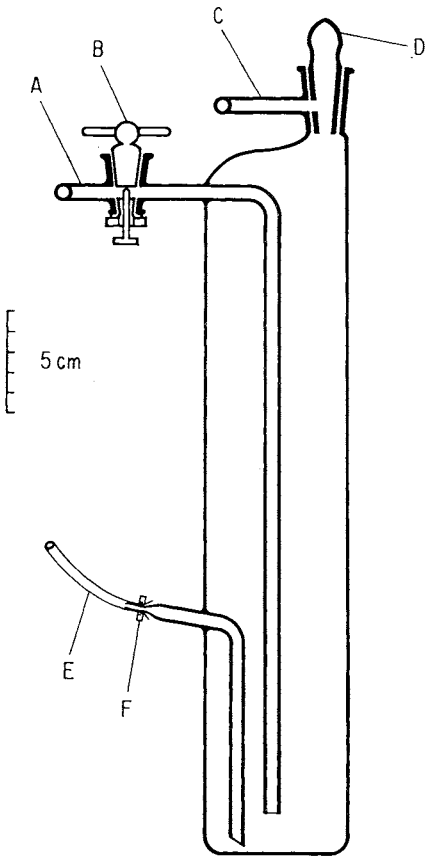


Fig. 4. *Reservoir.* *A*, nitrogen inlet; *B*, stopcock; *C*, nitrogen outlet; *D*, stopper (spring loaded); *E*, reagent (solvent) line; *F*, S.S. sealing nut

pieces of gold are threaded into stainless steel shafts linking the valves to the cores of the solenoids. The valves are springloaded by stainless steel coils to a pressure of 3 kg. The valve chambers are sealed off from the outside by PTFE membranes and rubber O-rings. The assembly is connected to the reservoirs by PTFE tubes (2.7 mm inner diameter). Liquid enters through a channel at the bottom of the valve chamber, and leaves at the center of the valve seat. Capillary channels from each valve converge on the center of the PTFE block, where they merge into a common channel connected to the feed line.

The rubber O-rings of the heptafluorobutyric acid valve tend to perish because of the acid vapors, and should be replaced at intervals.

The valves are operated by solenoids (Guardian Solenoids Inc., Santurce, Puerto Rico, type 4-D.C. with high temperature winding).

Reservoirs

Nitrogen is admitted to the bottom of the reservoir (Fig. 4) *via* a glass-PTFE stopcock provided with a needle valve. The stopper has an outlet, which is closed except when nitrogen is passed through a

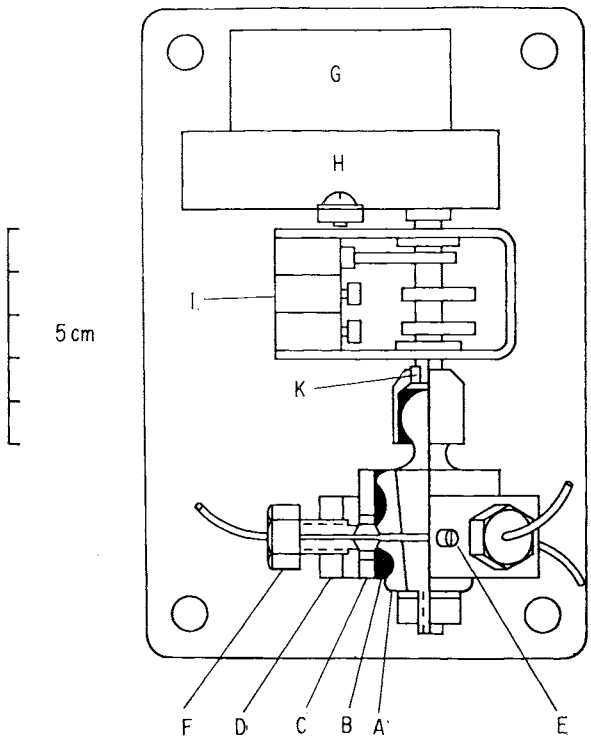


Fig. 5. *Outlet stopcock assembly.* *A*, 3-way stopcock; *B*, epoxy cement; *C*, inner S.S. ring; *D*, outer S.S. ring (fixed); *E*, aligning screw (one of three); *F*, sealing bolt; *G*, electric motor; *H*, gear box; *I*, set of cams and microswitches; *K*, sliding key on drive shaft

new batch of solvent or reagent in order to remove dissolved oxygen. The solvent and reagent reservoirs hold 1,200 ml and 150 ml respectively.

Outlet Stopcock Assembly

This has three positions (Fig. 5) which are termed: (a) 'vacuum', when the effluent line is closed; (b) 'collect', when the effluent line is connected to the fraction collector; and (c) 'waste', when the effluent line is connected to the waste container.

In addition to the obvious functions, two of these positions are used for other purposes. The waste

position is used when a reagent is being introduced into the cup, in order to prevent a buildup of pressure due to the evaporation of the reagent. The vacuum position is also used during the reaction stages, as otherwise the passage of nitrogen through the bell jar to the outside would cause evaporation of reagents in the cup.

A standard stopcock with a glass barrel and a PTFE key (Fischer and Porter Pty. Ltd., Melbourne, Australia, type 80G2417, 2 mm bore) has been so modified that it can be operated by an electric motor. The motor is synchronous, and provided with a gear box (Philips Electrical Ltd., Eindhoven, Netherlands, type: motor AU5100/22, gear box AU5300/80DJ). It is started by a signal from the programming unit, and stopped by either of three self-operated cam-and-microswitch combinations. The microswitches are hermetically sealed.

Fraction Collector

This is housed in a refrigerated stainless steel cabinet kept at $+2°$. The rack is made of stainless steel, and accomodates 50 tubes, each with a holding capacity of about 5 ml. The rack is operated by a suitably modified stepping motor of the uniselector type which is nonsparking.

The low temperature serves to minimize any decomposition of the unstable thiazolinones.

Pressure System

Two different pressures relative to the atmosphere are maintained (Fig. 1). The pressure in the bell jar, P_1, serves to assist the removal of liquid through the effluent line during extractions. P_1 is kept at 50 cm of water. A higher pressure, P_2, is maintained in the reservoirs. The latter are at a lower level than the cup, the bottom of the reservoirs being 36 cm below the bottom of the cup, and pressure is required to transfer liquid. A suitable rate of flow results when $P_2 = 150$ cm of water.

A pressure head is provided by a cylinder of compressed nitrogen equipped with a standard pressure reduction valve. On the other side of the reduction valve the line is divided in two branches, one to the bell jar and the other to the reservoirs. Each branch has a low pressure regulator and a pressure gauge. The pressure regulators are of the type commonly used for compressed butane burners (Commonwealth Industrial Gases Ltd., Melbourne, Australia, type AW51). These are well suited for their purpose since they may be accurately set, and permit a large flow of gas.

The pressure line to the bell jar joins the vacuum line at the 3-way valve (Fig. 1, M) in such a way that the bell jar may be connected either to the pressure line or to the vacuum line.

Vacuum System

Vacuum is supplied by a 2-stage rotary gas ballast pump with a pumping capacity of 30 l per minute and powered by a 3-phase induction motor (Rud Browne and Co. Pty. Ltd., Melbourne, Australia, type Dynavac 2) (Fig. 1). All lines and valves are made of stainless steel. The 3-way valve (The Skinner Chuck Co., New Britain, Conn., U.S.A., type V53DB2VAC2, coil 140 volts D.C.), connects the bell jar either to the vacuum line or to the pressure line. The former is the case when the solenoid is energized. The 2-way valve (The Skinner Chuck Co., type V52DB2077, coil 140 volts D.C.) has been so modified that it has a permanent bypass between the inlet and the outlet gates. The bypass is a coil of stainless steel tubing, length 32 cm and inner diameter 0.60 mm. When the bell jar is evacuated the 3-way valve opens immediately but the 2-way valve only after a delay period. During the delay period the system is therefore evacuated only through the bypass restriction. This arrangement serves to prevent the liquid in the cup from boiling through too sudden an evacuation.

The ultimate vacuum attainable in the system is approximately 10^{-2} mm of Hg. This degree of vacuum is ensurance that leakage from the atmosphere is sufficiently reduced. However, during actual operation this low pressure is not reached because of the contamination of the system with products of low volatility. The pressure observed at the end of a vacuum stage is usually not lower than $5 \cdot 10^{-2}$ mm of Hg.

Programming Unit

This unit (Fig. 6) is an electronic timer with 30 channels, each timing a stage in the degradation cycle. The channels are arranged in sequence so that each channel, when reaching a preset time, initiates the next, and the last channel triggers the first. The channels control, *via* relays, the various functions, *i.e.* valves and motors of the sequenator.

A set of three decade counters (X1, X10 and X100; Philips Electrical Ltd., type 88930/33), here referred to as the impulse counter, receives time pulses from a pulse generator (see below) *via* a pulse shaper (B; Philips Electrical Ltd., type 88930/48). Signals from the impulse counter operate sets of program stages (H; Philips Electrical Ltd., type 88930/37), which can be individually set for 1 to 999 counts.

The impulse counter is common to a number of program stages. The selection of the stage is made by a combination of a ring counter (0; Philips Electrical Ltd., type 88930/33 suitably modified), here called the stage counter, and a set of AND-gates (G), one for each program stage. One gate at a time is held open by the output from the stage counter according to its setting.

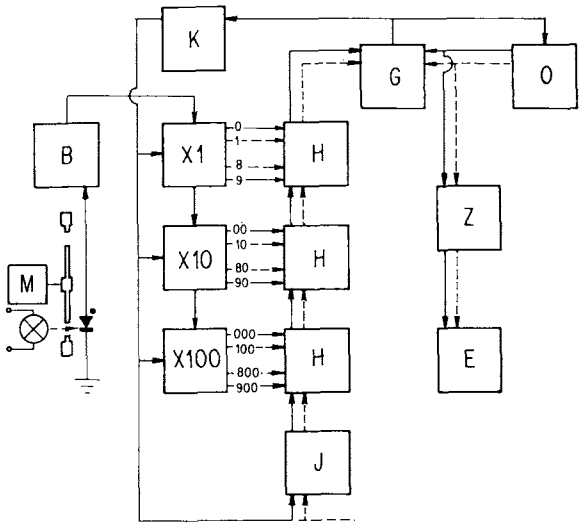


Fig. 6. *Diagram of programming unit.* *X* 1, *X* 10, *X* 100, impulse counters; *M*, pulse generator; *B*, pulse shaper; *H*, program stage; *O*, stage counter; *G*, AND-gate; *J*, program initiator; *K*, reset unit; *Z*, relay amplifier; *E*, output relay

Provisions are made for the manual: (a) resetting of impulse counters and program stages; (b) resetting of stage counter; (c) selection of stage; (d) interruption of pulse generator; and (e) interruption of output commands.

Time Pulse Generator. Light from a low voltage miniature light bulb falls on a photosensitive diode (OAP12). The light beam is interrupted by a rotating disc with two diametrically situated holes. The disc is driven by a synchronous motor (Philips Electrical Ltd., type AU5005/22) through a gear box (Philips Electrical Ltd., type AU5300/80DA). The impulse frequency is 20 per minute.

The sequenator is kept in a constant temperature room at 20 ± 2°.

REAGENTS AND SOLVENTS

The processes performed by the sequenator are essentially the same as those in the manual procedure [6]. The protein or peptide is first exposed to a coupling medium consisting of an alkaline buffer,

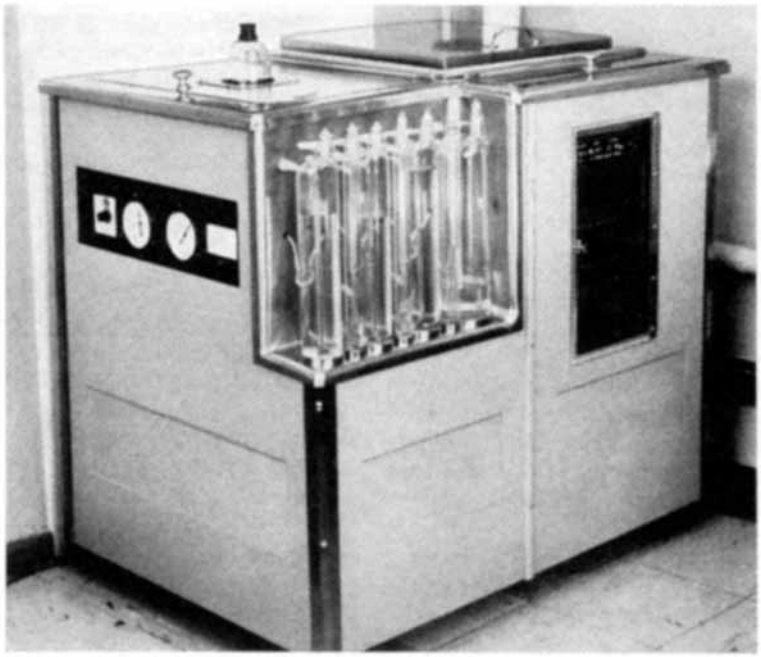


Fig. 7. *The sequenator*

The pulse allowed through a gate resets the impulse counter and the program initiators (J; Philips Electrical Ltd., type 88930/51), and also moves the stage counter to the next stage. The resetting pulse is modified by a reset unit (K; Philips Electrical Ltd., type 88930/54).

The signals from the stage counter operate the output relays (E) *via* relay amplifiers (Z; Philips Electrical Ltd., type 88930/57).

phenylisothiocyanate, and a solvent to form the phenylthiocarbamyl derivative. After completion of the reaction excess reagents and by-products are removed by solvent extraction, and residual solvent subsequently evaporated. The thiazolinone is then cleaved off by exposing the phenylthiocarbamyl derivative to an anhydrous acid, and is subsequently extracted by an organic solvent. After drying the shortened peptide is ready for a new degradation

Table. *Operations in a cycle of the sequenator*

Reagents 1, 2 and 3 are in reservoirs I, II and III respectively, and solvents 1, 2 and 3 occupy reservoirs IV, V and VI respectively. The valves are numbered correspondingly. Other functions are the 3-way vacuum-pressure valve (M), the 2-way vacuum valve (N), the 3-way outlet stopcock (E), and the motor driving the fraction collector (F). A + or a − sign means that the operating solenoid or motor is energized or deenergized respectively at the beginning of the stage. The + sign also means that a valve is open, in the case of valve M to the vacuum line. The three positions of the outlet stopcock are indicated by the letters c (collect), v (vacuum), and w (waste). The duration of stages refers to a reaction temperature of 50°

Stage	Duration	(Volume)	Function									
	min	(ml)	I	II	III	IV	V	VI	M	N	E	F
1. Reagent 1	0.15	(0.40)	+								+w	+
2. Reagent 2	1.75	(0.40)	−	+								−
3. Reaction	30.00			−							+v	
4. Restric. vacuum	3.00								+			
5. Vacuum	6.00									+		
6. Delay	0.05								−	−		
7. Solvent 1	5.00	(11.5)				+					+w	
8. Solvent 2	8.00	(23.5)				−	+					
9. Delay	1.00						−					
10. Restric. vacuum	3.00								+		+v	
11. Vacuum	6.00									+		
12. Delay	0.05								−	−		
13. Reagent 3	0.70	(0.23)			+						+w	
14. Reaction	3.00				−						+v	
15. Vacuum	1.50								+	+		
16. Delay	0.05								−	−		
17. Solvent 3	1.75	(5.5)						+			+c	
18. Delay	1.00							−				
19. Restric. vacuum	3.00								+		+v	
20. Vacuum	1.00									+		
21. Delay	0.05								−	−		
22. Reagent 3	0.70	(0.23)			+						+w	
23. Reaction	3.00				−						+v	
24. Vacuum	1.50								+	+		
25. Delay	0.05								−	−		
26. Solvent 3	2.25	(7.0)						+			+w	
27. Delay	1.00							−				
28. Restric. vacuum	3.00								+		+v	
29. Vacuum	6.00									+		
30. Delay	0.05								−	−		

cycle. However, the mode of operation of the sequenator requires some modification of the reagents. The large volume of the bell jar and the high temperature (50°) require less volatile reagents. Therefore, the volatile organic buffer base has been substituted by a nonvolatile organic base, *N,N,N′,N′*-tetrakis-(2-hydroxypropyl)-ethylenediamine (Quadrol), and the trifluoroacetic acid [7,8] has been replaced by heptafluorobutyric acid. The reagents and solvents described here are those used successfully in the degradation of myoglobin (see Applications: results).

Composition

Reagent 1 is a 5% (v/v) solution of phenylisothiocyanate in heptane. Phenylisothiocyanate is unstable in the completed coupling medium, and is therefore kept as a separate reagent. Reagent 2 is a 1.0 M Quadrol-trifluoroacetic acid buffer in *n*-propanol-water (3:4, v/v), pH 9.0 (glass electrode, 20°). Reagent 3 is anhydrous *n*-heptafluorobutyric acid. Solvent 1 is benzene. Solvent 2 is ethyl acetate containing 0.1% (v/v) acetic acid. Solvent 3 is 1-chlorobutane.

Purification

Much emphasis is laid on the purity of reagents and solvents. Special efforts have been made to remove traces of aldehydes since these tend to react with the terminal amino group, and thus cause a progressive fall in yield. Because of their importance the purification procedures are here given in some detail.

Phenylisothiocyanate (*purissimum* grade; Fluka A.G., Buchs, Switzerland) is distilled *in vacuo* at 1 mm of Hg, and the fraction boiling at 55° collected. The reagent is somewhat unstable on storage even in the cold, and is therefore distilled only as required.

N,N,N′,N′-tetrakis-(2-hydroxypropyl)-ethylenediamine (Quadrol, Wyandotte Chemicals Corp., Wyandotte, Mich., U.S.A.) is a practical grade reagent, and holds appreciable amounts of aldehydic impurities, which are difficult to remove. No proce-

dure has been found that removes these impurities completely. The most successful method found is based on chromatography on an anion exchange resin charged with sulfite ions [9]. Five hundred grams of Amberlite IRA-500 (British Drug Houses Ltd., Poole, England, standard grade) is converted into the free base with 5 N NaOH, washed with distilled water, and made into a column (75 cm × 10 cm^2). A solution of 11 g of SO_2 in 250 ml of distilled water is passed through at a flow rate of 5 ml per minute, and the column then again rinsed with water. Sixty grams of Quadrol is dissolved in distilled water to a volume of 250 ml, and passed through the column at the same flow rate. During this passage some displacement of sulfite ions down the column is apparent from a change in color of the resin. The effluent is concentrated in a rotary film evaporator, first on the water pump and finally on the rotary oil pump. This preparation is then distilled and redistilled in a molecular still (Jena Glasswork, Schott & Gen., Mainz, Germany, type 5593 mvz) at a distillation temperature of 120° and a pressure of 10^{-3} mm Hg. The resulting preparation is low in, but not entirely free of aldehydes (see Tollens' reaction).

n-Heptafluorobutyric acid (Minnesota Mining and Manufacturing Co., Saint Paul, Minn., U.S.A.) is first exhaustively oxidized at refluxing temperature with solid CrO_3, distilled off, and dried over $CaSO_4$. It is redistilled on a short Widmer column, and the fraction boiling at 119—120° collected.

Trifluoroacetic acid (Minnesota Mining & Manufacturing Co.) is purified in a similar manner.

Acetic acid (*purissimum* grade distilled from CrO_3; Fluka A.G.).

Heptane (practical grade) is shaken with several changes of concentrated H_2SO_4, washed in succession with distilled water, 10% (w/v) aqueous NaOH, and distilled water. This is followed by shaking in a mechanical shaker overnight with a 3% (w/v) aqueous solution of $KMnO_4$. The $KMnO_4$ is removed by washing with distilled water, the preparation dried over Na_2SO_4 and distilled.

Benzene (practical grade) is stirred over concentrated H_2SO_4 with several changes of acid, and then washed with distilled water. It is then shaken in a mechanical shaker overnight with a 3% (w/v) aqueous $KMnO_4$ solution. The $KMnO_4$ is removed by washing with distilled water, and the preparation dried over solid KOH. It is finally distilled on a 50 cm Widmer column, and the fraction boiling at 80—81° collected.

Ethyl acetate (practical grade) is stirred, first with 5% (w/v) aqueous Na_2CO_3 and then with a saturated aqueous $CaCl_2$ solution. The organic phase is drawn off, and shaken overnight in a mechanical shaker with solid $KMnO_4$ (1 g/l). The organic phase is drawn off, washed with distilled water, dried over $CaSO_4$ and distilled, b.p. 77°.

1-Chlorobutane is a laboratory grade reagent (Hopkin and Williams Ltd., Chadwell Heath, Essex, England), and is shaken in a mechanical shaker overnight with a 3% (w/v) aqueous $KMnO_4$ solution. The $KMnO_4$ is removed by repeated washing with distilled water, and the preparation then dried over $CaSO_4$. It is distilled, and the fraction boilling at 78° collected.

n-Propanol is a laboratory grade reagent (May and Baker Ltd., Dagenham, Essex, England), and is refluxed for several days with powdered Zn (5 g/l) and saturated aqueous NaOH (5 ml/l), and then dried over $CaSO_4$. The preparation is fractionated on a 50 cm Widmer column, the fractionation being followed with Tollens' reaction. Usually only the last 1/3 of the distillate gives a negative reaction.

Nitrogen contains less than 10 ppm of oxygen (Commonwealth Industrial Gases, Ltd.).

Water is glass distilled.

Tollens' Reaction

Solvents and reagents are tested with this reaction for the absence of aldehydes wherever practicable (heptane, benzene, ethyl acetate, 1-chlorobutane, *n*-propanol, Quadrol). The test is carried out in a darkened room and in the following way. One milliliter of a 10% (w/v) aqueous $AgNO_3$ solution and 1 ml of a 10% (w/v) aqueous NaOH solution are mixed in a test tube, and a dilute aqueous NH_3 solution added dropwise until the precipitate is redissolved. To this solution is added 2 ml of the sample (Quadrol and *n*-propanol are first diluted 1:1 (v/v) with distilled water), and, if the solutions are immiscible, the tube is shaken at frequent intervals. No discoloration or cloudiness should appear within one hour, except in the case of Quadrol where a slight grayish discoloration indicates that some aldehydic impurities are still present. (Caution: Explosive silver fulminate is formed in the Tollens' reaction.)

OPERATION

The programming of the degradation cycle requires that it be divided in 30 stages. The stages, their durations and associated operations are shown in the Table, and only a few comments are required.

The vacuum stages 4, 5, 15, and 24 serve to concentrate or to remove the reaction medium prior to extraction. This has been found necessary since otherwise protein material tends to be carried up into the groove causing blockage of the effluent line. This is caused by the fact that at first the concentration of the extracting organic solvent is not high enough to precipitate the protein. The Quadrol offers special difficulties in this respect since it cannot be removed by evaporation. Instead, it is extracted by a solvent, benzene, with which is it miscible to a

limited extent. The extraction is subsequently completed by ethyl acetate.

During the restricted vacuum stages 4, 10, 19, and 28 the 2-way valve (Fig. 1, N) is closed, and evacuation is only through the capillary bypass.

The delay stages 9, 18, and 27 are inserted between an extraction stage and a vacuum stage. Their function is to allow time for a stream of nitrogen (the outlet stopcock is in the waste or collect position) to clear the effluent line and groove of solvent. This prevents splashing of solvent when vacuum is applied.

The delay stages 6, 12, 16, 21, 25, and 30 occur between a vacuum stage and a stage where a reagent or a solvent is added. They serve to allow pressure equilibration in the bell jar.

Before use all reagents and solvents are purged of dissolved oxygen by passing a lively stream of nitrogen through the reservoir for about five minutes.

The sample (0.2—1.5 μmoles) is introduced in the following way. The programming unit is manually operated (see Programming Unit) so that the pulse generator and the outgoing commands are interrupted, and the stage counter set at stage 27. The bell jar is removed, and 0.2 ml of a solution of the sample, *e.g.* in water or aqueous ethanol, is deposited (it is convenient to use an all-glass syringe with a short piece of PTFE capillary tube attached to the tip) low on the walls of the spinning cup. After replacing the bell jar, the programming unit is allowed to take over the operation. It is preferable to start from stage 27 rather than from stage 1 since this ensures that the system is purged of oxygen before the degradation is started.

The volumes of buffer and heptafluorobutyric acid are so adjusted in relation to each other that the buffer rises about 3 mm higher on the wall of the cup than the acid. This helps to confine the protein to the lower part of the cup.

The dry protein can be observed at the end of the stages 11, 20, and 29. It should then have a dry, powdery appearance, and be confined to the lower $^1/_3$ of the cup.

It is convenient to use a stroboscope for observing the processes taking place in the cup.

The time required for one degradation cycle is 93.6 minutes, which is equivalent to 15.4 cycles in 24 hours. The volumes of the reservoirs allow about 50 degradation cycles to be run without attention.

APPLICATION

The sequenator has been applied to the degradation of apomyoglobin obtained from the muscles of the humpback whale *(Megaptera nodosa)*.

Materials and Methods

Myoglobin was prepared from the skeletal muscles of the humpback whale by fractionation with basic

Fig. 8. *Degradation of apomyoglobin.* Thin layer chromatographic identification using solvent system D of the amino acid-PTHs obtained in first 60 degradation cycles. Reference mixtures are indicated by horizontal bars, and show in order from bottom to top the migration of (—) proline-PTH, valine-PTH, alanine-PTH, glycine-PTH; (=) leucine-PTH, methionine-PTH; (≡) isoleucine-PTH, phenylalanine-PTH and tryptophan-PTH

lead acetate and ammonium sulfate [10]. The filter cake was dissolved in distilled water, and potassium ferricyanide added to the solution (10 mg/g of filter cake). After removal of the salts by dialysis against distilled water and subsequent concentration of the solution, the preparation was further fractionated through chromatography on CM-cellulose [11]. A column of CM-cellulose (27 cm $\times$ 10 cm^2) was equilibrated against a 0.01 M Tris phosphate buffer, pH 8.00. The column was charged with 1 g of the Fe(III)-myoglobin preparation, and developed with the same buffer at $+4°$ and with a flow rate of 50 ml per hour. The main peak, constituting about 85% of the material, appeared well resolved at an elution volume of 900 ml, but there were several smaller, partly resolved peaks earlier in the chromatogram. The main fraction was collected, dialysed against distilled water, and concentrated to approx. 10 g/l. The haem was removed by the acetone-hydrochloric acid procedure [12]. After dialysis, first against a dilute $NaHCO_3$ solution and finally against distilled water, the preparation was freeze-dried.

The conversion of the thiazolinones into PTHs was carried out somewhat differently from what has been described earlier (3). The present conversion conditions are N aqueous HCl, 80°, and 10 minutes. These conditions gave a considerably better recovery of serine-PTH without any disadvantageous effect on the other amino acid-PTHs. After evaporation of the 1-chlorobutane solution of the thiazolinone to dryness, 0.2 ml of N HCl was added, the tube flushed with nitrogen, stoppered and kept at 80° for 10 minutes. After cooling the sample was extracted with altogether 3 ml of ethyl acetate. The organic phase was evaporated to dryness, and the residue taken up with brief heating in a suitable volume of ethylene chloride (approx. 5 μmoles of PTH per ml). This solution was used for chromatography. The aqueous phase if the sample contained arginine-PTH or histidine-PTH, was evaporated to dryness *in vacuo* over pellets of KOH and P_2O_5, and the dry residue taken up in a suitable volume of distilled water for electrophoretic identification.

The amino acid-PTHs were identified by thin layer chromatography or electrophoresis on silica gel containing an ultraviolet fluorescent indicator (Fluka A.G., type D5F). The paper chromatographic systems D and E [13] could be applied directly to thin layer chromatography. The spots were located either through the extinction of fluorescence under the low pressure mercury lamp, or through the iodine-azide-starch reaction. Arginine and histidine-PTHs were identified by thin layer electrophoresis in an apparatus and with a technique similar to that described by Honegger [14]. The electrophoresis was carried out in a 0.01 M sodium phosphate buffer, pH 8.0, for one hour at a field intensity of 10 volts per cm. The silica gel is acid in itself, and it is necessary to neutralize it beforehand. This was done by making up the slurry of silica gel used for coating the plate in the same buffer, and drying the plate in the air overnight. The electrophoretic buffer was sprayed on to the plate by the normal procedure.

Results

The degradation was performed on 5.0 mg of the apomyoglobin preparation, and was carried through 60 cycles. The yield was determined from the ultraviolet absorption at 269 mμ of the amino acid-PTHs in cycles Nos. 1 and 17 (both valines), and the average yield for a single cycle was calculated to be 98.5%. The results of the thin layer chromatography of the amino acid-PTHs using solvent system D are shown in Fig. 8 (see page 89). These results, together with those obtained by using solvent system E and thin layer electrophoresis, permitted the deduction of the following N-terminal structure for humpback whale myoglobin:

Humpback Whale: Val.Leu.Ser.*Asp.Ala.*Glu.Trp.Gln.Leu.Val.Leu.*Asn.Ile.* Trp.Ala.Lys.Val.Glu.Ala.Asp.
Sperm Whale: Val.Leu.Ser.*Glu. Gly.* Glu.Trp.Gln.Leu.Val.Leu.*His. Val.*Trp.Ala.Lys.Val.Glu.Ala.Asp.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Val.Ala.Gly.His.Gly.Gln.Asp.Ile.Leu.Ile.Arg.Leu.Phe.Lys.*Gly.*His.Pro.Glu.Thr.Leu.
Val.Ala.Gly.His.Gly.Gln.Asp.Ile.Leu.Ile.Arg.Leu.Phe.Lys.*Ser.*His.Pro.Glu.Thr.Leu.
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

Glu.Lys.Phe.Asp.*Lys.*Phe.Lys.His.Leu.Lys.Thr.Glu.Ala.Glu.Met.Lys.Ala.Ser.Glu.Asp.
Glu.Lys.Phe.Asp.*Arg.*Phe.Lys.His.Leu.Lys.Thr.Glu.Ala.Glu.Met.Lys.Ala.Ser.Glu.Asp.
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

The corresponding part of the structure for sperm whale myoglobin [15] is presented for comparison, and the non-identical amino acids are italicized.

DISCUSSION

The usefulness of a sequenator process depends in the first place on the yield in the single degradation cycle. A simple calculation will illustrate this point. Let it be arbitrarily assumed that a sequence determination ceases to give useful information when the overall yield, *i.e.* the yield over n degradation cycles, has fallen to 30%. It may then be calculated that an average repetitive yield, *i.e.* the yield from one cycle to the next, of 97, 98 and 99% would allow 40, 60 and 120 cycles respectively. In the present work the

repetitive yield has been 98% or slightly better. The cause of the loss of about 2% in each degradation cycle is at present not known. However, it should be emphasized that even a moderate reduction of this loss would allow a considerable extension of the degradation.

Another important factor in the termination of a degradation has been the appearance in the chromatograms of an increasing general background of other amino acid-PTHs, which eventually made the identification impossible. What causes this background is not certain. It may be significant that the dominating amino acids in the background are also those which occur most frequently in the structure, *e.g.* glycine, alanine, and leucines in the case of myoglobin (Fig. 8). A small degree of nonspecific cleavage along the peptide chain would produce this result. A mechanism is at hand to account for a nonspecific cleavage, since it has been shown that anhydrous trifluoroacetic acid may cause acidolysis of peptide bonds [16], and heptafluorobutyric acid would be expected to behave in the same way. However, in the absence of direct evidence, this explanation for the appearance of the background remains tentative.

An incomplete reaction either during the coupling or the cleavage would produce overlap between consecutive steps. Even if this occurred only to a small degree in each cycle, the overlap would become quite apparent after many cycles because of the cumulative effect. An incomplete reaction has in fact been observed during the cleavage step, and has been particularly noticable for aspartic and glutamic acid, but much less so for the amino acids with nonpolar side chains. It appears that the incomplete cleavage is due not to an insufficient reaction time, but rather to an equilibrium being reached before complete cleavage has occurred. The incompleteness of the cleavage reaction is the reason for its repetition in the present procedure. This has reduced the overlap to an insignificant level even in extended degradations.

With the limitations already discussed it is possible to identify without ambiguity every amino acid split off. However, it should be pointed out that, due to decompositions during the conversion reaction (3), asparagine, glutamine, and serine produce spots in addition to those of the expected PTH. Asparagine and glutamine-PTHs are partly hydrolysed to aspartic acid and glutamic acid-PTHs, and serine-PTH is partly converted into unidentified decomposition products. Another question is if the prolonged exposure of the protein to the conditions of the degradation could produce alterations in the amino acid residues *per se*. This has so far not been observed. Tryptophan is here of particular interest since it is known to be decomposed by anhydrous acids [17]. However, it should be noted that tryptophan does not occur after position 14 from the N-terminal end of the myoglobin structure.

The conditions of degradation which have been described here are those which have been found suitable for myoglobin, and also for a limited number of other proteins. However, it may be necessary to modify the conditions to suit other cases. Special difficulties are likely to be encountered with short peptides. The small difference in solubility between the thiazolinone and the short peptide makes difficult the differential extraction of the former, and yields therefore tend to fall off rapidly as the degradation approaches the C-terminal end of the peptide. This problem is at present being investigated.

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