

Apparatus for Solid-Phase Peptide Synthesis

Abstract. *The apparatus, designed for solid-phase peptide synthesis, consists of a round-bottom flask, rocked on a wrist-shaker, and fitted with a special dropping funnel and a fritted filter disc embedded within the flask. The dropping funnel is designed to wash down the polymer adhering to the neck of the flask. Solvents are removed through the fritted disc. Entire synthesis, and the removal of peptide from the polymer, are carried out without opening or removing the vessel from the shaker.*

A new approach to the synthesis of peptides, termed solid-phase peptide synthesis, was introduced recently by Merrifield (1). This method was based on the idea that a peptide chain could be lengthened in a stepwise process while it was attached at the C-terminal end by an ester bond to an insoluble polymer, and that it could be liberated in the form of a free peptide after the desired sequence had been assembled. The merits of this method have already been discussed by Merrifield (2). Apart from the fact that this method solves the technical difficulties associated with solubility and purification of pep-

tide chains of intermediate length the new approach considerably accelerates the coupling procedures and allows all the chemical reactions to be carried out in a single "suitable" vessel. This report concerns this latter aspect.

Merrifield and Stewart constructed an automatic apparatus (3) for this purpose, which appears to be very useful for the synthesis of large peptides and proteins, but is perhaps too elaborate and expensive for many laboratories dealing with the synthesis of small peptides. It therefore appeared of interest to construct a simple apparatus of wide applicability.

Merrifield's manually operated vessel (1) (Fig. 1) consists of a 45- by 125-mm glass cylinder, sealed at one end and fitted with a 40-mm, medium-porosity disc filter at the other end. A side-arm, fitted with a drying tube, was used to introduce reagents and to remove solid samples for analysis. In order to provide mixing of solvents and polymer, the apparatus was attached to a mechanical rocker, which rotated the vessel 90° between

the vertical and horizontal positions. At the end of each reaction, the vessel was stopped in the vertical position with the fritted disc at the bottom, so that opening the stopcock allowed the solvents to be removed by suction. However, this apparatus suffers from the following disadvantages:

(i) It is inconvenient and time-consuming to open the drying tube or stopper every time solvents or reagents are added to the reaction vessel and to wash in the polymer adhering to the neck of the vessel. This difficulty is well appreciated if one realizes that the operation is repeated at least 30 to 35 times during each coupling.

(ii) The rocker (4) for this purpose is rather slow and requires more time for complete mixing.

(iii) The elongated construction and the rotation of the vessel 90° between the vertical and horizontal position does not allow complete washing and mixing of the polymer adhering to the upper side of the vessel (side A in Fig. 1). This difficulty perhaps can be overcome by utilizing smaller vessels.

Rudinger (5) suggested that a conical reaction chamber, with one fritted filter disc at the top and the other at the bottom, may be constructed, and solvents and reactants may be continuously circulated through the chamber. Consequently, an apparatus (Fig. 2) modified from the suggestion of Rudinger was constructed. This apparatus had the advantage of continuous oper-

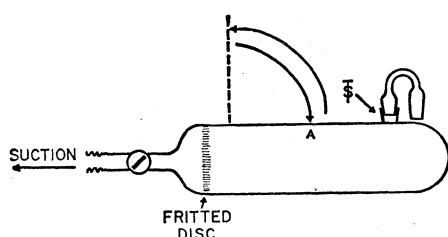


Fig. 1. Merrifield's manually operated vessel.

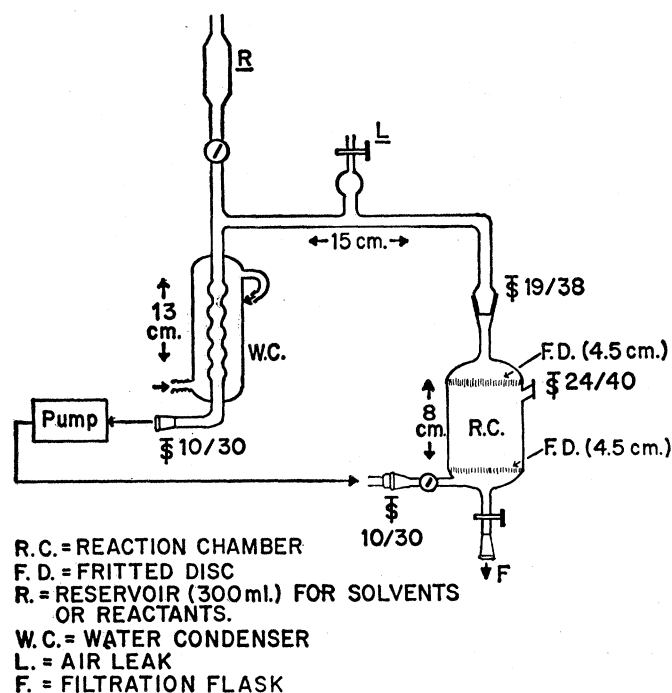


Fig. 2 (left). Apparatus based on Rudinger's suggestion.

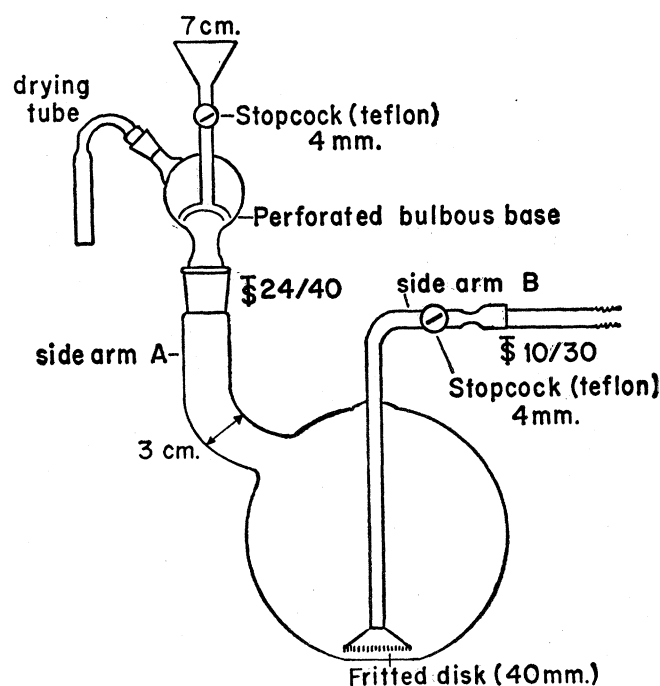


Fig. 3 (right). Our new apparatus for solid-phase peptide synthesis.

ation, but was found to have many disadvantages:

(i) Fritted glass discs became clogged with dicyclohexylurea or triethylamine hydrochloride salt during the continued recycling of the reactants.

(ii) The reaction chamber was not very flexible to different quantities of polymer. This was especially noticeable because of the swelling of the polymer in such solvents as dimethylformamide (DMF) or methylene chloride. Similarly, one could not use large volumes of solvents to dissolve intermediates with low solubility in DMF, for example, *tert*-butyloxycarbonyl-nitroarginine or *tert*-butyloxycarbonyl-*N*^{lm}-benzyl-L-histidine.

(iii) To avoid channeling flow, reversible flow was required, and a special circulating pump (peristaltic pump, Sigma-Motors, model TL) was essential. Further, suitable tubing for circulating both acids and solvents, such as DMF, is not available. "Tygon" was found to be suitable for acid conditions and "silastic" for DMF and methylene chloride, but it is inconvenient to change the tubing. Polyethylene tubing could not be used on this pump because it lacked proper flexibility.

(iv) Mixing was slower than in conventional shaking, and it was necessary to allow more time for mixing.

In view of these difficulties, we decided to construct an apparatus as illustrated in Fig. 3. It consisted of a 250- or 500-ml round-bottom flask, flattened at the bottom and fitted with two side arms. To side arm A was fitted, through a standard 24/40 joint, a bulb carrying a drying tube as an air-leak and a dropping funnel; the two octapeptides, L-alanine³-L-isoleucine⁵-angiotensin II (6, 7) and L-isoleucine¹-L-isoleucine⁵-angiotensin II (7, 8), were synthesized with this apparatus in overall yields of 55 to 60 percent. However, low yields or impure products are not due to a faulty coupling or a faulty apparatus but to the final step in which the peptide is removed from the polymer. The best available procedure for the cleavage of peptide from the polymer requires the passage of hydrogen bromide through a suspension of the peptide-polymer in trifluoroacetic acid. Exposure for 15 to 20 minutes results in 50 to 60 percent yields of the angiotensin peptides, while exposure for 1 hour or more to obtain quantitative cleavage yields a complex mixture of products not hydrolyzable by leucine aminopeptidase (9)

to the component amino acids. This final step warrants further investigation to improve the procedure of angiotensin synthesis.

Our apparatus appears to be very convenient. During the synthesis of the octapeptides it was found that the solvents or reagents could be transferred very conveniently, the polymer adhering to the sides of the side arm A could be washed in easily, mixing of the polymer and removal of solvents was very efficient, the flask could be immersed in ice water with simultaneous shaking for carrying out the coupling at low temperature, and the entire synthesis was carried out without opening or removing the flask from the shaker. For cleavage of peptide from the polymer, the peptide-polymer was washed twice with glacial acetic acid followed by trifluoroacetic acid. The polymer was then suspended in trifluoroacetic acid and a slow stream of hydrogen bromide bubbled through the fritted disc into the suspension.

The polymer was filtered and washed with trifluoroacetic acid, and the peptide was obtained from the filtrate in the usual manner.

MAHESH C. KHOSLA*

ROBERT R. SMEBY

F. MERLIN BUMPUS

Research Division, Cleveland Clinic Foundation, Cleveland, Ohio 44106

References and Notes

1. R. B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149 (1963).
2. ———, *Endeavour* **24**, 3 (1965); *Biochemistry* **3**, 1385 (1964).
3. R. B. Merrifield and J. M. Stewart, *Nature* **207**, 522 (1965); R. B. Merrifield, *Science* **150**, 178 (1965).
4. Chipco Mfg. Co., San Bruno, Calif.
5. J. Rudinger, personal communications.
6. M. C. Khosla, R. R. Smeby, F. M. Bumpus, *Biochemistry*, in press.
7. F. M. Bumpus, M. C. Khosla, R. R. Smeby, to be presented at the American Chemical Society meeting, April 1967.
8. M. C. Khosla, R. R. Smeby, F. M. Bumpus, in preparation.
9. G. R. Marshall and R. B. Merrifield, *Biochemistry* **4**, 2394 (1965).
10. Our thanks are due to Professor Merrifield for helpful discussions. Part of this work was supported by NIH research grant No. H6835.
- * Present address: Central Drug Research Institute, Lucknow, India.
- 11 January 1967

Creatine Phosphokinase in Thyroid:

Isoenzyme Composition Compared with Other Tissues

Abstract. Considerable creatine phosphokinase activity was demonstrated in thyroid glands of several species. After starch gel electrophoresis, thyroid exhibited a creatine phosphokinase isoenzyme pattern different from that of muscle, heart, and brain. The isoenzyme repertory of various tissues and a hybrid enzyme produced from muscle and brain enzyme are consistent with the postulated dimeric structure of creatine phosphokinase.

In addition to those tissues (brain, and skeletal and cardiac muscle) previously reported to be rich in creatine phosphokinase (1), we have found significant concentrations of this enzyme in the thyroid gland of several species including man (2), confirming the finding of Hess *et al.* on a small sample of human thyroids (3). The creatine phosphokinase activity of thyroid tissue of various species is listed in Table 1.

Separation of isoenzymes was accomplished by starch gel electrophoresis. Aqueous homogenates (1:5 for muscle, heart, and brain; 1:3 for thyroid) of freshly obtained rabbit tissues were centrifuged at 100,000g for 1 hour and the supernatants were electrophoresed in borate buffer, pH 8.0, ionic strength 0.12, with a current of 5 volt/cm for 16 hours. The gel was repeatedly washed with 0.2M glycine buffer, at pH 6.75, because the subsequent staining reaction proceeds

poorly at the higher pH. Creatine phosphokinase activity on the gel was demonstrated with the following reaction mixture: creatine phosphate $7.6 \times 10^{-3}M$, adenosine diphosphate $1.1 \times 10^{-3}M$, Mg acetate $1.0 \times 10^{-1}M$, glucose $3.3 \times 10^{-2}M$, nicotinamide adenine dinucleotide phosphate $9.2 \times 10^{-4}M$, nitroblue tetrazolium $5.9 \times 10^{-4}M$, phenazine methosulfate $1.4 \times 10^{-4}M$, hexokinase 490 international units per 100 ml, glucose-6-phosphate dehydrogenase 420 international units per 100 ml, prepared in 1.0M glycine buffer.

This linked reaction is a modification of Oliver's method (4) and results in deposition of a blue formazan at the sites of creatine phosphokinase activity in the gel. Adenylic kinase also yields formazan. Omission of creatine phosphate from the reaction mixture often revealed a faintly stained slow band of adenylic kinase.