Studies on the Biosynthesis of Rabbit Fibrinogen

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The attention of this Laboratory has recently been centred on the synthesis of serum albumin by subcellular preparations of rat liver. In considering what other plasma protein synthesized in the liver might be studied in this way, fibrinogen offered many advantages. It was first shown by Miller, Bly & Bale (1954) that fibrinogen was synthesized in the dog solely in the liver, a finding recently substantiated by Kukral, Kerth, Pancner, Cromer & Henegar (1961). Moreover, the rate of synthesis of fibrinogen as deduced from its metabolic turnover is probably greater than that of any of the other groups of serum proteins. For example, Cohen, Holloway, Matthews & McFarlane (1956) estimated that the half-life of fibrinogen in the rabbit is 52–85 hr., compared with 190 hr. for serum albumin.

From structural considerations fibrinogen is also a protein of much interest. Unlike albumin, which is a globular protein, fibrinogen is a fibrous protein, and the mode of synthesis of the two groups may be different (Gerber & Altman, 1961). Moreover, in the conversion of fibrinogen into fibrin under the action of thrombin at least two peptides are released, the removal of one of which, designated peptide A, appears to be essential for the subsequent polymerization of fibrin [for review see Laki, Gladner & Folk (1960)]. Although previous experiments with isotopically labelled amino acids failed to provide definite evidence of the existence of peptides as intermediates in protein synthesis (Campbell, 1958), a protein with the properties of fibrinogen has not previously been studied. Blombäck, Boström & Vestermark (1960) have shown that the half-life of peptide B in the rabbit is similar to that of fibrin.

It was decided therefore to study the rate of incorporation of an amino acid into the molecule of fibrin and to compare this with the rate of incorporation of the same amino acid into peptide A. Blombäck & Sjöquist (1960) have shown that in the rabbit the single valine residue in peptide A occupies the *N*-terminal position, although this was not known when the present experiments were started. It is also known that the fibrin moiety contains a high proportion of valine residues

(Tristram, 1953); so it was decided to inject rabbits with [³H]valine and to determine the specific radioactivity of the valine in peptide A and fibrin after a short time-interval. It was hoped thereby to learn something of the metabolic activity of the peptide A compared with that of the rest of the molecule. A preliminary account of this work has appeared (Craddock & Campbell, 1961).

MATERIALS AND METHODS

Radioactive amino acids. Uniformly labelled L-[¹⁴C]valine (100 μ C/1.8 mg.), an acid hydrolysate of ¹⁴C-labelled algal protein (100 μ C/2.7 mg.) and DL-[³H]valine (5 mC/29.25 mg. used in Expt. I and 5 mC/22.13 mg. in Expt. II) were obtained from The Radiochemical Centre, Amersham, Bucks.

Injection of animals and isolation of fibrinogen. Rabbits of approx. 3 kg. (body wt.) were injected via a marginal ear vein with DL-[³H]valine dissolved in 0.067 M-phosphate buffer, pH 7.3. After 20 min. the animals were anaesthetized by injection of phenobarbital sodium, and bled from the inferior vena cava through a silicone-treated tube into a vessel containing sodium oxalate and potassium oxalate. The blood of the two animals was pooled. The plasma was fractionated according to 'Method 6' of Cohn *et al.* (1946), and the percentage of 'clottable nitrogen' of fraction I was determined by the method of Biggs & MacFarlane (1957).

Isolation of fibrin and peptide A. Fraction I was dissolved in ammonium acetate (0.3 M, pH 7.0) and clotted by the method of Blombäck *et al.* (1960). The fibrin clot was separated from the supernatant (which was retained), cut up, and washed ten times with water (5 ml. each time). The washings failed to give a positive reaction with ninhydrin after five operations. The washings were rejected and the fibrin was dried with ethanol.

The clot supernatant was adjusted to pH 3.0 by the addition of 10 N-formic acid, and peptide A was isolated by chromatography according to the method of Blombäck *et al.* (1960). In this procedure a column (0.9 cm. $\times$ 30 cm.) of Dowex 50 (2% cross-linked; 200–400 mesh) is eluted stepwise first with ammonium formate–formic acid buffers, and then with ammonium acetate–acetic acid buffer. Peptide A was detected in the eluent by the method of Lowry, Rosebrough, Farr & Randall (1951). The peptide solution was desalted by adsorption on a small column of Dowex 50 (2% cross-linked; H⁺ form), and eluted with aq. 2 N-ammonia and evaporated to dryness.

Isolation of valine from fibrin and peptide A. The fibrin clot was dried with ethanol and a portion (65 mg.) hydrolysed in a sealed tube with 1.5 ml. of 5 N-HCl at 110° for

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24 hr. The humin was removed by centrifuging and the amino acids were separated on a column (0.9 cm. $\times$ 100 cm.) of Zeo-Karb 225 (W.R. 1.5–2.0; < 200 mesh) according to the method of Hirs, Moore & Stein (1954), with increasing concentrations of HCl as eluent. After the emergence of alanine from the column the concentration of HCl was increased from 1N to 2N. The amino acids in the effluent were located by paper chromatography in butan-1-ol-acetic acid-water (12:3:5, by vol.). The fractions containing valine were pooled and evaporated to dryness, and the purity of the product was checked by paper chromatography.

Peptide A was hydrolysed and the hydrolysate treated in the same way as for fibrin, except that as the amount of valine present was too small to estimate directly an isotope-dilution procedure was used. L-Valine (300 μ g.) was added as carrier together with 0.5 μ C of L-[14 C]valine. The specific radioactivity of the valine isolated from the column was determined by plating samples on 2 cm.² polythene disks and counting under a thin mica end-window Geiger-Müller counter at infinite thinness to a standard error of $\pm 3\%$ or less. The amount of valine on the disks was determined by interaction with ninhydrin according to the method of Cocking & Yemm (1954). The value obtained was compared with that of a sample of 0.5 μ C of L-[14 C]valine plus 300 μ g. of L-valine treated in the same way. The amount of valine originating in the peptide was then calculated.

Determination of the specific radioactivity of [3 H]valine. Samples of valine (30–300 μ g.) were 'spotted' on strips of Whatman no. 1 filter paper, so that the total area of the spot did not exceed 2 cm.². The area of the spot was then cut out and the [3 H]valine determined by combustion, followed either by reduction with Zn to H₂ gas, or by direct counting of the 3 H₂O by liquid-scintillation counting in the Packard Tricarb Automatic Liquid Scintillation spectrometer, by the method of Jacobson, Gupta, Fernandez, Hennix & Jensen (1960). The counting efficiency was 12.1% as determined with a standard prepared from 3 H₂O obtained from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Preliminary experiments in which a rabbit was injected with a mixture of 14 C-labelled amino acids obtained from algal protein showed that the plasma proteins were significantly labelled 20 min. after injection. Since the maximum incorporation into plasma protein in the rabbit is found after about 6 hr. (Campbell & Work, 1952), it was decided to limit the time of incorporation to 20 min. so that any non-uniformity in the distribution of labelled amino acid would be apparent. The results obtained in two experiments are given in Table 1. The 'clottability' of fraction I was 79%. Contaminating protein was separated from peptide A during its chromatographic isolation, and any protein adhering to the fibrin clot was removed during the repeated washing. By assuming a molecular weight of 350 000 for rabbit fibrinogen (Schulman, 1953), and that the peptide A contains one valine residue (Blombäck & Sjöquist, 1960), the yield of valine

Table 1. *Incorporation of [3 H]valine into fibrinogen in vivo*

	Expt. I	Expt. II
Dose of [3 H]valine/rabbit (mc)	2.5	5.0
Dry wt. of fibrin (mg.)	230	243
Amount of valine obtained from peptide A (μ g.)	40	69
Specific activity of peptide valine	64.5	74.6
Specific activity of fibrin valine	8.4	18.7

originating in the peptide was approx. 50%, similar to that obtained by Blombäck *et al.* (1960).

In each experiment the specific activity of the peptide valine was considerably higher than that of the valine originating in the fibrin. The difference between the two experiments may be due to the increased specific activity of the valine injected in the second experiment, or to the increased amount of valine administered. In either case more fibrinogen would have been synthesized before the specific radioactivity of the valine fell to a low level.

DISCUSSION

The results show that the valine incorporated into fibrinogen synthesized in a short time-interval by the liver *in vivo* is not uniformly distributed throughout the molecule. The higher specific activity of the peptide valine may be taken as evidence that the N-terminal peptide is synthesized separately from the remainder of the molecule, and is turned over at a greater rate. An alternative explanation would be that growth of the fibrinogen chain occurs by the sequential linking of amino acids, terminating in the region of the free amino end of peptide A. Then, assuming that partly formed fibrinogen molecules were present at the time of injection, the remaining amino acid sites would be filled with labelled valine, and the higher specific activity of this region would be expected.

With haemoglobin there is evidence that the amino acids are added to the polypeptide chain sequentially, starting at the N-terminal end (Bishop, Leahy & Schweet, 1960) and ending at the free carboxyl end (Dintzis, 1961).

To investigate these two possibilities it would be necessary to know the distribution of [3 H]valine within the fibrin. Experiments involving characterization of the peptides formed on hydrolysis of tritiated fibrin should give information on this.

SUMMARY

1. The distribution of [^3H]valine in fibrinogen synthesized by the rabbit in a short time-interval *in vivo* was investigated. The valine located in peptide A was found to have a higher specific activity than that from the remainder of the molecule.

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Studies in the Biochemistry of Micro-organisms

110. PRODUCTION AND BIOSYNTHESIS OF ORSELLINIC ACID BY *PENICILLIUM MADRITI* G. SMITH*

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A study of the metabolism of *Penicillium madriti* G. Smith led to the isolation from the culture filtrate of two metabolic products, penicillic acid and orsellinic acid. Penicillic acid, a fairly common mould metabolite, was first isolated from *Penicillium puberulum* Bainier by Alsberg & Black (1913). Orsellinic acid had not previously been recognized as a fungal product, although Aghoramurthy & Seshadri (1954) had postulated it as an intermediate in mould metabolism. In view of the work of Birch, Massy-Westropp & Moye (1955) on 6-methylsalicylic acid, to which orsellinic acid is structurally closely related, acetate coupling seemed the most obvious pathway for biosynthesis, particularly since orsellinic acid, $\text{C}_8\text{H}_8\text{O}_4$, can

theoretically be derived from four acetate units without oxidation or reduction. We have now verified this hypothesis and investigated the possibility of a C_4 intermediate such as acetoacetic acid in the metabolic pathway between acetate and orsellinic acid. A fuller account of this work is given by Gowlland (1961).

While this investigation was in progress, Mosbach (1959) reported the isolation of orsellinic acid from the culture filtrates of *Chaetomium cochliodes* Palt. and showed that when supplied with $\text{CH}_3\cdot^{14}\text{CO}\cdot^{18}\text{ONa}$ the fungus produced orsellinic acid in which the labelling of both carbon and oxygen atoms conformed to the requirements for a direct condensation of activated acetate units to orsellinic acid (Gatenbeck & Mosbach, 1959). Later work by Mosbach (1960) has demonstrated

* Part 109: Birkinshaw & Gourlay (1961).