methylbutan-1-ol and centrifugation to separate the emulsion, all the labelled DNA is precipitable from the resulting aqueous layer with ethanol. However, it was found that not all the DNA is subsequently recoverable by the classical 'spooling' technique and moreover a large proportion remained insoluble in 0.15M-sodium chloride. This is referred to as 'residual DNA'.

Treatment of this insoluble fraction with α -amylase followed by mild alkali was sufficient to solubilize completely a fraction sensitive to the digestive actions of pancreatic deoxyribonuclease, snake-venom diesterase and spleen diesterase. Although sucrose-density-gradient analyses revealed this DNA species to be of low molecular weight (approx. 5S), the kinetics of its labelling before and after a 'chase' indicated it to be metabolically stable. Also, like the remainder of the nuclear DNA, it is replicated during the S-phase of the cell cycle.

Perhaps the most interesting feature of this 'residual DNA' species was revealed by chromatography on columns of hydroxyapatite. Despite the solubilization procedures, which involve alkali treatment, the 'residual DNA' still behaves in a fashion expected of a double-stranded molecule. Attempts to 'denature' the material either by heating at low ionic strength followed by rapid cooling, or by treatment with alkali followed by neutralization, were unsuccessful. One possible explanation of such behaviour is that the molecule is somehow cross-linked. So far only pretreatment with 2-mercaptoethanol has rendered the molecule 'denaturable'. Nevertheless after this treatment the DNA is still readily 'renatured' by incubation at high ionic strength, suggesting the presence of repetitious nucleotide sequences.

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Initiation of Deoxyribonucleic Acid Replication in Mammary-Gland Organ Cultures

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When explants from the mammary glands of mid-pregnant C3H mice are cultured in medium 199 with insulin the parenchymal cells are induced to replicate their DNA and divide (Stockdale, Juergens & Topper, 1966; Mayne & Barry, 1970). We have found that the same changes occur when medium 199 is replaced by a simpler medium containing only inorganic salts, glucose and amino acids. Withdrawal and replacement of the components of this medium have enabled us to study the substrates required for the initiation of DNA replication.

We conclude that the initiation and synthesis of DNA in organ culture, as measured by the incorporation of [3 H]thymidine, can be divided into three distinct periods. At 0–4h there is a lag period in which insulin, glucose and amino acids may be absent from the medium. At 4–12h there is a period in which these components must be present and that ends by the subsequent rise in DNA synthesis being insensitive to actinomycin D. At 12–22h there is the period in which parenchymal cells are in S-phase. DNA synthesis in this period is unaffected by the absence of insulin, glucose or amino acids from the medium or by inhibition of RNA synthesis, but it is immediately stopped when protein synthesis is inhibited by cycloheximide.

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Effect of Azaserine on Insulin Release *in vitro*

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Azaserine (O-diazoacetyl-L-serine), an analogue of glutamine, has been shown to act *in vivo* to inhibit markedly the synthesis of purine nucleotides *de novo* in various mammalian tissues (Moore & Le Page, 1957). It has been suggested (Mayhew, Wright & Ashmore, 1969) that purine nucleotides may be involved in the metabolic events leading to insulin release from the mammalian pancreas in response to various secretagogues. On this basis it was decided to determine whether azaserine could exert an effect *in vitro* on the release of insulin from isolated rat islets of Langerhans. A parallel study was undertaken with mouse islets of Langerhans in view of reported species differences in the effects of other inhibitors.

Islets of Langerhans were prepared by collagenase digestion of the pancreas (Howell & Taylor, 1968) and were preincubated for 30 min in a physiological medium containing 5 mM-glucose. After a further incubation for 30 min in the presence of glucose or 8 mM-glucose plus the agent under study the medium was sampled and the insulin content was determined by radioimmunoassay.

With rat islets of Langerhans no effect of 0.3 mM-azaserine was observed on the rate of insulin release in response to 20 mM-glucose or the release produced by 5 mM-leucine, 5 mM-theophylline or 1 mM-dibutyryl cyclic AMP (6-N,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate. However, the

potentiation of release caused by 5mM-arginine was completely abolished. Also, the rate of release in response to a new secretagogue, 20mM-D-xylulose, was significantly diminished.

In studies with mouse islets of Langerhans the rate of release in response to 20mM-glucose was, however, markedly inhibited, as were the effects of 5mM-arginine. The effect of 5mM-leucine was not completely inhibited, and again there was no decrease in the rate of release due to 5mM-theophylline.

Although there may clearly be species differences in the response to azaserine, these results further suggest that the formation of purine nucleotides may be important in the insulin secretory process.

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Separation of Bovine α - and β -Trypsin by Affinity Chromatography

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Affinity chromatography has been used for the selective purification of enzymes (Cuatrecasas, Wilchek & Anfinsen, 1968), and the removal of inert protein from bovine trypsin by affinity chromatography on avian ovomucoids covalently linked to Sepharose has been reported (Feinstein, 1970a,b). Bovine trypsin has been resolved into two major active components, α - and β -trypsin, by ion-exchange chromatography (Schroeder & Shaw, 1968). We report convenient procedures for the isolation of these enzymes by affinity chromatography.

A potent competitive inhibitor of trypsin, *p*-(*p*'-aminophenoxypropoxy)benzamidine (Baker & Erickson, 1968), was coupled at pH 5.0 via the terminal amino group to Sepharose 4B (Pharmacia) and to cellulose powder (Whatman CC31) by using a cyanogen bromide activation procedure (Cuatrecasas *et al.* 1968). The products generally contained 0.6–1.0 μ mol of inhibitor/ml of packed polysaccharide. Trypsin was assayed with the spectrofluorometric titrant 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride as described by Roberts, Adams, Elmore, Jameson & Kyle (1971). The two forms of trypsin were identified by titration with 4-methylumbelliferyl *p*-guanidinobenzoate

(about 1 μ M) at pH 8.3, since β -trypsin was acylated in a few seconds, but α -trypsin required over 10 min.

A solution of trypsin (lot TRL 81B; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) (20mg) in triethanolamine–50mM-HCl–10mM-CaCl₂ buffer, pH 8.0, was applied to a cellulose-inhibitor column (2.2cm $\times$ 14.3cm) at 4°C, and a peak of inert protein was immediately eluted. Subsequent elution with 50mM-sodium acetate–acetic acid, pH 5.0, yielded a peak of pure β -trypsin (5.1mg). Elution with 50mM-citric acid–50mM-NaCl, pH 2.3, then released all the remaining trypsin (5.2mg), a mixture of the two forms. Gradient elution failed to resolve these. The isolation of β -trypsin was adversely affected by 0.5M-NaCl in the triethanolamine buffer, and by the use of ammonium formate buffers.

When a solution of trypsin (20mg) in 50mM-sodium acetate–acetic acid, pH 4.0, was applied to the cellulose inhibitor column at 4°C, most of it was immediately eluted in a single peak. When, however, the enzyme (50mg) in the same buffer was applied to a Sepharose-inhibitor column (1.5cm $\times$ 25cm) at 4°C, inert protein, α -trypsin and β -trypsin were successively eluted as three incompletely resolved peaks. Selected fractions contained α -trypsin (3.8mg) and β -trypsin (12.4mg). A mixture of the two trypsins (3.4mg) remained bound to the column, and was eluted by 0.1M-citric acid–1M-NaCl, pH 2.0.

Factors such as type of support material, buffer composition, pH and ionic strength strongly influence these separations and will be discussed.

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The Kinetics of Hydrolysis of Synthetic Substrates by Bovine Factor Xa

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Bovine Factor Xa, thrombokinase and autoprothrombin C may be identical (Esnouf & Williams, 1961; Marciniak & Seegers, 1962; Milstone,