thiaminase I[8,9]. In addition, since the decrease in the enzyme activity was not observed when hydroxymethylpyrimidine or hydroxyethylthiazole was added to the growth medium (data not shown), it was presumed that thiamine or thiamine pyrosphosphate accumulated in cells acts as a corepressor to repress thiaminase II synthesis in *S. cerevisiae*.

Table 4 shows the effect of pyrithiamine and oxythiamine on the growth of *S. cerevisiae* and a hydroxyethylthiazole kinase deficient mutant of *S. cerevisiae*[4]. 5 ml of thiamine-free Wickerham's synthetic medium with or without thiamine antagonist was inoculated with a washed cell suspension of *S. cerevisiae* or its mutant equivalent to 0.016 mg (dry weight) and incubated at 30 °C without shaking. Growth after 18 h was then measured turbidimetrically at 560 nm. It was found that the reversal of pyrithiamine-induced growth inhibition of *S. cerevisiae* by oxythiamine was impaired in a hydroxyethylthiazole kinase deficient mutant of *Saccharomyces cerevisiae*. The results show that in *S. cerevisiae* enzymes synthesizing thiamine from hydroxymethylpyrimidine and hydroxyethylthiazole[5] are essentially involved, in addition to thiaminase II in the production of thiamine from pyrithiamine and oxythiamine, resulting in the relief of growth inhibition by these two thiamine antagonists.

In conclusion, it was demonstrated that *Saccharomyces cerevisiae* contains thiaminase II together with thiamine-synthesizing enzymes. It is well known that yeast not only synthesizes thiamine de novo but takes up and accumulates thiamine from the medium. The possible role of thiaminase II in the regulation of thiamine metabolism in *S. cerevisiae* remains to be clarified.

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Synthesis of an analog of human calcitonin gene related peptide, [Asu^{2,7}]-h-CGRP

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Summary. The analog of h-CGRP, des-Ala-deamino-dicarba-h-CGRP, was synthesized by the combination of the conventional solution and the solid phase peptide synthesis methods. This analog showed stronger and longer-lasting hypocalcemic and hypophosphatemic activities than the natural hormone.
Key words. h-CGRP analog; synthesis; Ca and Pi lowering effects.

Calcitonin Gene Related Peptide (CGRP) was identified in 1983 as an alternative gene product of the calcitonin gene in the rat by Rosenfeld et al.[1]. In man, complementary DNA encoding the precursor of CGRP was recognized by Steenbergh et al.[2] in messenger RNA extracted from medullary thyroid carcinoma. The amino acid sequence of human CGRP was also derived from medullary thyroid carcinoma extracts[3]. Subsequently, additional CGRP genes were identified in rat and man by Rosenfeld et al.[4] and Steenbergh et al.[5], respectively. The peptides all consist of 37 amino acids with disulfide bridges between Cys 2[6] and Cys 7, and their remaining amino acid sequences are highly homologous.

Several biological effects of CGRP have been reported including hypocalcemia and hypophosphatemia[7], hypotension, an increase of heart rate[8] and inhibition of gastric acid secretion[9].

Tippins et al.[10] have reported that destruction of the disulfide bridge at the amino-terminus abolished biological activity, but the modification of the middle and carboxyl-terminal regions of this hormone (such as acylation of Lys 24 or Lys 35 and substitution of Val 22 and Asn 25 with Met and Ser, respectively) did not alter the biological activity. Therefore, the amino-terminal portion, especially the S-S linkage of the peptide, appears to be important for biological activity.

The present paper deals with the synthesis of an analog in which the amino-terminal amino acid(Ala) and the amino group of Cys 2, and the S-S bond of the natural peptide were replaced by a hydrogen atom and by an ethylene linkage, respectively (fig. 1).

Recently, solid phase peptide synthesis has made it possible to synthesize peptides of 30 to 40 amino acids very rapidly. However, a peptide which contains a fragment linked with ethylene instead of the S-S bond is much more troublesome to synthesize by this method. Therefore, the synthesis of the analog was carried out using a conventional solution method for the preparation of amino-terminal fragment [1], which corresponded to the sequence (2–8) of the natural hormone. Fragment [1] was coupled to the solid phase carboxyl-terminal linear fragment (9–37)-resin. All the functional groups in the amino acid residues were protected by groups which are used commonly in solid phase synthesis. Fragment [1] was synthesized as shown in figure 2. Each fragment was purified by the conventional procedures, and applied to the next step after confirming the homogeneity by thin layer chromatography and by amino acid analysis. Compound [5] was converted to the p-nitrophenyl ester at the ω-position of the Asu residue with p-nitrophenyl trifluoroacetate[11]. After removal of the amino protecting group (Boc), [4] was treated with pyridine at 50 °C under high dilution condition (1 m mol/l). The crude cyclized product [3] was treated with anhydrous HF at 0 °C for 1 h in the presence of anisole, followed by column chromatography on CHP-20P (Mitsubishi Chem-

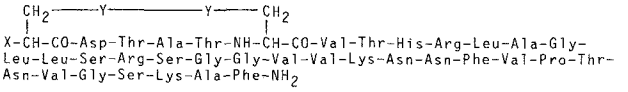

Figure 1. Structure of h-CGRP (X = Ala, Y = S) and [Asu^{2,7}]-h-CGRP (X = H, Y = CH_2).

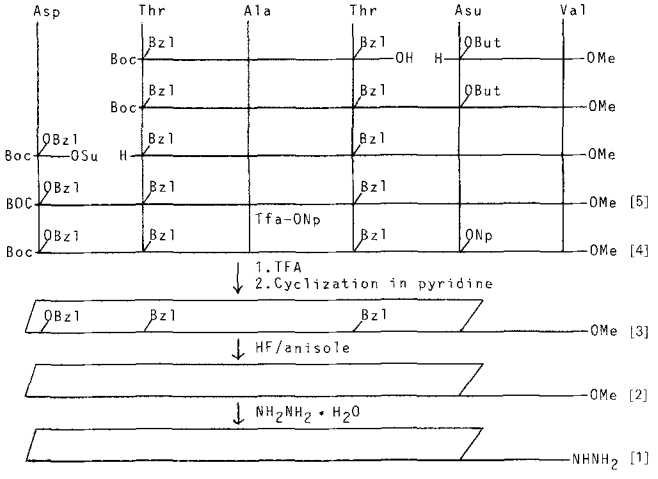

Figure 2. Synthesis of fragment [1].

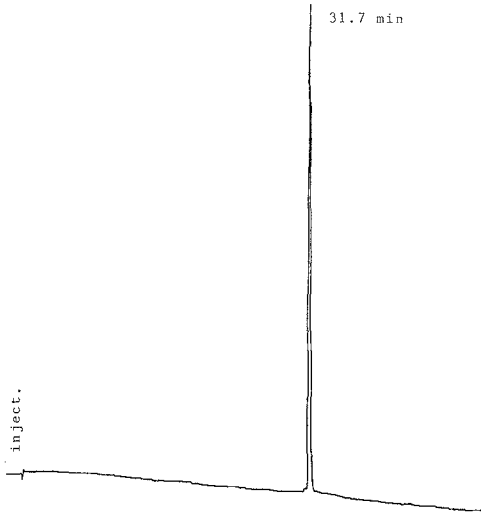

Figure 3. HPLC of the [Asu^{2,7}]-h-CGRP

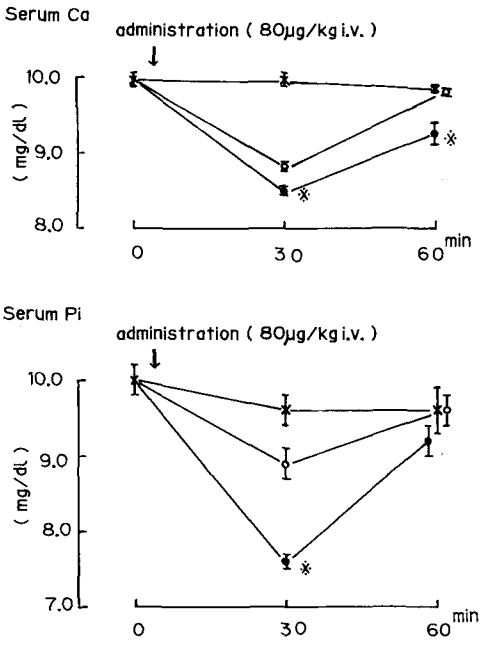

Figure 4. Serum calcium and phosphate lowering effects of h-CGRP and [Asu^{2,7}]-h-CGRP. ×——×, control; ○——○, h-CGRP; ●——●, [Asu^{2,7}]-h-CGRP. Each point represents the mean ± SE (n = 5). ·Ẋ· indicates significant difference from h-CGRP with $p < 0.05$.

ical Industries, Ltd.) using gradient elution with DMF (20% to 66%) in 0.1 M AcOH to obtain the methylester [2] which exhibited a single peak in analytical reverse phase HPLC (33% overall yield from the cyclization). The cyclized fragment was confirmed by its molecular ion peak (M^+673) in the mass spectrum.

The carboxyl terminal fragment (9–37)-resin was synthesized using an automatic solid phase synthesizer '430-A Peptide Synthesizer' (Applied Biosystem Inc.). The protocols using single coupling (except for Arg, Gln and Asn which were double-coupled) were implemented throughout the syntheses. To p-methylbenzhydrylamine resin, the 37th to 9th amino acids were coupled successively to obtain the fragment (9-37)-resin. For each synthesis cycle, quantitative ninhydrin monitoring was done by implementing a modified version of a protocol published by Sarin et al.[12]. The average step yield was 98.0%. After deprotection of the α-amino group, the peptide-resin was coupled with the fragment [1] by the azide method. The obtained protected peptide(2-37)-resin was treated with anhydrous HF as mentioned above, and the liberated free peptide was purified by the use of CM-cellulose column chromatography, using a linear gradient concentration of an ammonium acetate buffer (0.02 M to 0.2 M) at pH 6.0, then CHP-20 P column chromatography using linear gradient concentration of acetonitrile (20% to 40%) in 0.1 M of acetic acid, and finally by the reversed phase HPLC of Nucleosil $_5C_{18}$ followed by gradient elution with acetonitrile (10% to 50% in 40 min) in 0.1% TFA. A peak eluted at 31.7 min was collected and lyophilized to give the pure h-CGRP analog (fig. 3). Thus, homogeneous [Asu^{2,7}]-h-CGRP was obtained in an overall yield of 10% through the final coupling and purification procedures. This peptide has the following physical properties, $[\alpha]_D^{26.5}$ −46° (C=0.087, 0.1 M AcOH). pI > 10.25. The ratio of amino acids in an acid hydrolysate; Asp 4.08(4), Thr 3.77(4), Ser 2.74(3), Pro 1.1.(1), Gly 4.15(4), Ala 1.00(1), Val 4.65(5), Leu 3.20(3), Phe 2.18(2), Lys 2.11(2), His 0.98(1), Arg 2.10(2), Asu 1.12(1).

For the measurements of the hypocalcemic and hypophosphatemic activities, 80 μg/kg of the analog was administered to male Wister rats (80–90 g b. wt) through the tail vein. After 30 and 60 min, the serum calcium and phosphate concentrations were measured by procedures outlined by Kumar et al.[13] and by Goldenberg et al.[14]. This analog showed stronger serum calcium and phosphate lowering effects than the natural hormone (h-CGRP) (fig. 4). Moreover, the calcium-lowering effect of the analog lasted significantly longer. These findings and Tippins's results indicate that the disulfide bond in CGRP is essential not for the biological activity but for maintenance of the specific conformation of the molecule. The analog here described has no α-amino group, therefore it should be more resistant to degradation by α-amino peptidase than the native hormone. This would be one reason why the analog showed longer-lasting activity in vivo.

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6 Amino acids except Gly denote the L-configuration. Abbreviations are in accordance with the recommendations of IUPAC-IUB Commission [J. biol. Chem., *247* (1972) 977]. Bzl, Benzyl; Boc, t-Butyloxycarbonyl; Z, Benzyloxycarbonyl; HOSu, N-Hydroxysuccinimide; HONp, p-Nitrophenol; Asu, L-amino suberic acid; TFA, Trifluoroacetic acid; DMF, Dimethylformamide; HPLC, High performance liquid chromatography.
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The synthesis of stereoselectively labelled porphobilinogen and its incorporation into protoporphyrin-IX

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Summary. 11-(R)-^{2}H porphobilinogen, stereospecifically labelled with deuterium in the aminomethylene group has been incorporated into protoporphyrin-IX by haemolysates of chicken erythrocytes. High field NMR spectroscopy confirms that the overall biochemical process is stereospecific, deuterium being retained at the α-, γ- and δ-*meso* positions and lost from the β-*meso* position.
Key words. Porphobilinogen; stereoselectively deuterium-labelled protoporphyrin-IX; biosynthesis from porphobilinogen.

Some years ago we showed[1] that the dehydrogenation of protoporphyrinogen-IX **1** to protoporphyrin **2** in chicken red-cell haemolysates was a stereospecific process, but these experiments did not enable us to deduce the precise nature of the process, e.g. the stereochemistry of the hydrogen removal at each *meso*-bridge. Similar conclusions were reached in experiments in which doubly labelled (^{14}C, ^{3}H) porphobilinogen was incubated with a cell-free system from *Euglena gracilis*[2]. More recently Akhtar and Jordan have described[3] studies with tritium-labelled glycine which have effectively shown that both the enzymic formation of the macrocycle and the subsequent dehydrogenation process must be stereospecific.

We now describe experiments on the chemical synthesis of stereospecifically labelled porphobilinogen intended as a preliminary to further studies of the mechanism and stereochemistry of the in vivo formation[4] of the porphyrinogen macrocycle, and of related chemical processes. The route chosen to synthesise porphobilinogen was based on the original method worked out in MacDonald's laboratory from a suitably substituted pyrrole[5]. Following model experiments with simple alkyl pyrroles, the pyrrole aldehyde **3** related to porphobilinogen was converted into the imine **4** by condensation with R(+)-1-phenylethylamine. This was reduced with diborane and hydrolysed to give the corresponding amine **5a**, and with perdeuterodiborane followed by hydrolysis to give the mono-deutero analogue **5b**. Comparisons of the 360 MHz proton NMR spectra of the deuterated and undeuterated materials (fig. 1) showed that the latter was stereoselectively labelled (ca 40% e.e.). Attempts to improve the degree of specificity were of little avail. However, the stereoselectively deuterated phenylethylaminomethyl pyrrole **5b** was hydrogenolysed over palladium on charcoal to the corresponding aminomethyl pyrrole tri-acid **6** (with retention of stereochemical integrity). The latter was then converted[5] via the lactam **7**, decarboxylation and hydrolysis to deuterium-labelled porphobilinogen **8**.

The absolute stereochemistry of our deuterium-labelled porphobilinogen has been deduced as R by conformational analysis. There is a considerable body of evidence in the literature[6] that α-formylpyrroles have a preference for the *syn*-conformation as shown in structure **3**, and a priori it may be concluded that related imines also have a similar preference as shown in structure **4**. This was confirmed by the n.O.e. difference spectra of several imines of this type which

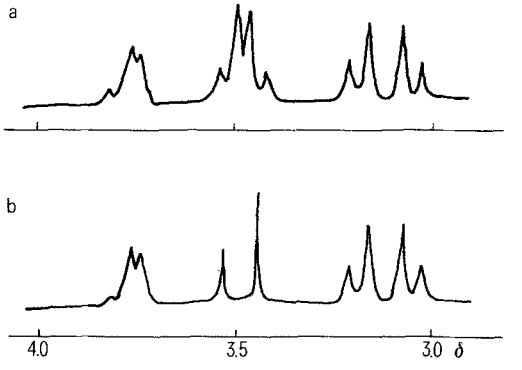

Figure 1. Partial 360 MHz proton N.M.R. spectra showing the resonances circa δ 3.5 of the α-aminomethylene groups in *a* the unlabelled pyrrole (**5a**) (an AB quartet), and *b* the stereoselectively deuterium-labelled pyrrole (**5b**) (two singlet resonances, ratio 7:3, corresponding to the two diastereoisomers).