thione inhibited the peroxidase activity at 10^{-3} *M*. Over and above the reagents mentioned in the text above compounds are added in a final concentration as mentioned.

Whether these peroxidases are iodide peroxidase or not was assayed according to the method of ALEXANDER[14]. 150 µmoles of sodium phosphate buffer pH 5.5, 20 µmoles of KI, 2.4 µmoles of H_2O_2 and an appropriate amount of enzyme in a total volume of 3 ml. Optical density was then measured at 353 nm. Figure 2 shows that this head kidney soluble supernatant peroxidase significantly oxidized iodide into tri-iodide. Formation of I^{-3} at 353 nm suggest that the enzyme is active in the oxidation of iodide which means that thyroid activity may be located in the soluble supernatant portion of the head kidney. As the thyroid of this fish is very diffused and ill-developed, and as some earlier reports conclude the inactivity of thyroid gland in fishes[15,16], this iodide oxidation is very significant.

Zusammenfassung. In der Kopfniere des Teleostiers *Anabes testudineus* wurde eine besonders hohe Peroxidase-Aktivität nachgewiesen, insbesondere deren Iodid-Oxydation zu Tri-Iodid.

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Collagen Biosynthesis in vitro and the Consequences of Bacterial Contamination. Studies on Vascular Connective Tissue in Rabbits

The collagen biosynthesis in connective tissue of various organs has been studied extensively in the last decade. One of the methods used for this purpose has been the measurement of the synthesis of ^{14}C-hydroxyproline from ^{14}C-proline, as completely described for the skin by UITTO[1].

Normally, antibiotics are added to incubation systems in vitro to eliminate a possible effect of bacterial contamination. To the authors knowledge, however, no systematic studies have been published on the effect of bacteria on the analytical system. Furthermore, an effect of added antibiotics per se on the biosynthesis of collagen and elastin cannot be excluded.

The aim of the present investigation was to compare the results obtained under sterile conditions and those in the presence of bacteria.

Material and methods. Male albino rabbits, about 5 months old and weighing about 3 kg, were fed a commercial chow and sacrificed by an animal pistol. The hair of the abdominal wall was washed with 60% ethanol, whereupon the skin was cut with a sterile pair of scissors.

Sterile conditions: With a new sterile pair of scissors, the thorax and abdomen were opened and the aorta was dissected free with a third set of sterile instruments. The aorta was immediately transferred to a sterile Krebs-Ringer-Bicarbonate solution placed in a sterile 1 m^3 incubator through which a laminar air flow was led. All the following manipulations were done within this sterile incubator. The intima-media part of the aorta was isolated as described by LORENZEN[2], and then sliced parallel to the axis of the aorta in eight 0.8 mm broad strips using parallel razor blades and a manual vice.

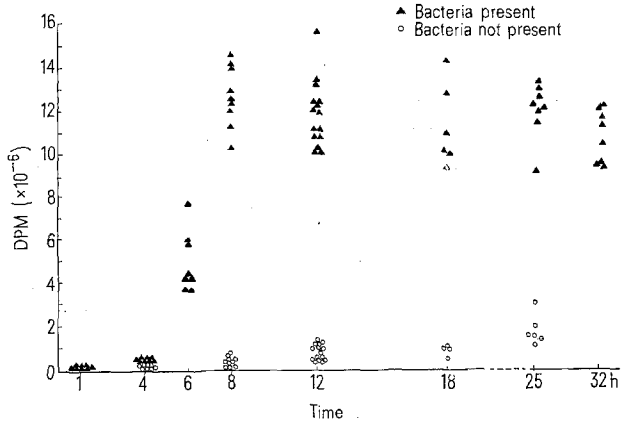

Fig. 1. DPM ^{14}C-proline incorporation in intima-media of rabbit aorta per 2000 µg hydroxyproline as function of incubation time. △, Bacteria present; ●, Bacteria not present.

The tissue-slices were transferred to sterile tubes containing 2 ml of a Krebs-Ringer-Bicarbonate medium[3], 20 m*M* N-2-hydroxy-ethylpiperazine-N^1-2-ethanesulphonic acid (Hepes) pH 7.4, 20 m*M* glucose and 2.5 µCi L-^{14}C-proline (New England Nuclear Cooperation. Radiochemical purity greater than 99.5%. Uniformly labelled).

The tubes were aseptically stoppered, placed in a waterbath and shaken at 37 °C for 12 h unless otherwise stated. The time taken from when the animal was shot until the tubes were stoppered was 4–5 min. At the end of the incubation period samples of the incubation medium were removed for bacterial examination in a bacteriological laboratory. The incubation tubes were stored at –20 °C until biochemical examination, which was performed up to 2 weeks later.

Bacterial conditions: The same procedures as described above were used except that only 1 pair of scissors was used and that the incubator was not used. No bacteria were added to the sterile incubation medium and the treatment of the tissue could best be described as 'as sterile as possible'. Bacterial examinations were done with one inoculation stick from each of the samples at the end of the incubation time, as well as from the ^{14}C-proline batch and the remaining incubation medium. All inoculation sticks were taken to a bacteriological laboratory[4] and numerous amounts of bacteria were taken as an index of contamination.

After storage at –20 °C, all samples were treated equally as follows. The samples were homogenized in 13 ml water in a VirTis '45' at high speed for 10 min. The temperature surrounding the vial was kept at –6 to –8 °C, thus keeping the temperature inside the sample at about +2 °C.

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Aliquots were taken from the homogenate for determination of total hydroxyproline and α-amino nitrogen. The remainder was dialyzed overnight against cold running tap-water, hydrolysed, evaporated, dissolved in water and examined for total ^{14}C-activity according to PROCKOP and EBERT[5], and for ^{14}C-hydroxyproline according to JUVA and PROCKOP[6] with the following modifications: 1. 0.25 *M* chloramin T was used instead of 0.2 *M* because in separate assays it was found that the recovery was maximal using 0.25 *M*, 2. after passing through the columns only 15 ml of the toluene were counted in a scintillator vial giving higher counting efficiency. All vials were counted in a Beckmann LS 250.

When analysing for ^{14}C-hydroxyproline in the presence of large amounts of ^{14}C-proline, some of the measured radioactivity is due to ^{14}C-proline[6]. Correction for this was made by subtracting 'blind values' from observed values. In each experiment blind values were determined by adding ^{14}C-proline to hydrolysed nonradioactive samples in amounts which corresponded to the total ^{14}C-activity of the ^{14}C-proline incubated samples.

In order to determine the optimum pH, the incorporation of ^{14}C-proline and synthesis of ^{14}C-hydroxyproline was measured at different pH values – from pH 6.6 to 8.2. All the incubations were done under sterile conditions with a 12 h incubation period to test the validity of the incubation system.

Total hydroxyproline and α-amino nitrogen. Samples for determination of hydroxyproline as well as α-amino nitrogen were hydrolysed in 6 *N* HCl at 136 °C for 6 h. The hydroxyproline was analysed by the JASIN and ZIFF method[7] and the α-amino nitrogen according to the RUBINSTEIN and PRYCE method[8]. All samples were run in duplicate.

If protein:hydroxyproline ratio in one sample from a series varied more than 2 S.D. from the mean, the analysis of α-amino nitrogen and hydroxyproline was repeated.

Results. Figure 1 shows that in the presence of bacteria the incorporation of ^{14}C-proline into rabbit aortas was 10–15 times higher after 12 h of incubation than under the sterile conditions, and that a tremendous ^{14}C-proline uptake takes place between 4 and 8 h of incubation. This was in contrast to the continuously progressing incorporation seen under sterile conditions.

The formation of ^{14}C-hydroxyproline under sterile conditions reached a plateau after 12 h (Figure 2). The slight decline which seemed to occur after 18 h was insignificant ($P < 0.2$). These values were lower at all incubation periods ≥ 8 h than those found in the presence of bacteria. When bacteria were present, the values found after 12 h of incubation were higher than those found after shorter or longer incubation.

The type of bacteria present in the experiments varied from experiment to experiment, but less so from sample to sample within one experiment. The Gramnegative bacteria always dominated and more than 2 types were regularly present in the same sample. Bacterial contamination was found neither in the ^{14}C-proline batches nor in the incubation medium before the incubation.

The Table shows incorporation and hydroxylation of ^{14}C-proline in sterile aortic slices after 12 h of incubation with different pH in the incubation system. At pH 7.4 both incorporation and hydroxylation were maximal.

Discussion. Bacterial contamination increased both the incorporation and hydroxylation of ^{14}C-proline in the in vitro analyses of the collagen and elastin biosynthesis in the aortic wall. That the incorporation was elevated was not surprising since proline is ubiquitous also in bacterial proteins[9]. But as hydroxyproline has been isolated only from a few bacterial strains[9], it might be possible that the

Incubation pH	Per cent ± S.E.M. of the pH 7.4 value	
	Incorporation (*n*)	Hydroxylation (*n*)
6.6	9.3 ± 1.0 (4)	10.9 ± 3.6 (4)
7.0	59.4 ± 13.1 (4)	53.2 ± 13.3 (6)
7.2	78.6 ± 25.2 (5)	76.4 ± 20.3 (6)
7.4	100.0 ± 6.9 (11)	100.0 ± 6.1 (11)
7.6	72.8 ± 14.8 (6)	65.7 ± 16.1 (6)
7.8	54.5 ± 7.4 (6)	64.6 ± 12.2 (6)
8.2	18.5 ± 6.6 (5)	14.9 ± 4.6 (6)

Incorporation of ^{14}C-proline and hydroxylation of ^{14}C-proline (^{14}C-hydroxyproline) in intima-media of rabbit aorta per 2000 μg hydroxyproline at different pH. Incubation time 12 h. Bacteria not present. At pH 7.4 the incorporation value was 555.000 dpm. The hydroxylation value was 12.100 dpm.

^{14}C-hydroxyproline was synthetized in the aortic slices. Since ^{14}C-hydroxyproline synthesis after 12 h were significantly higher with bacterial contamination than without, it seems possible that bacteria can accelerate the ^{14}C-hydroxyproline synthesis in rabbit aorta. OLSSON and NECHELEZ[10], MEIER[11] and JACKSON et al.[12] have found that bacterial pyrogens increased the production of collagen. Recently CASTOR[13] has reported that in rheumatic tissues the collagen biosynthesis was stimultated by Gramnegative bacteria but not by Grampositive.

The declining ^{14}C-hydroxyproline values after 12 h of incubation in the presence of bacteria is taken as an index of degradation of hydroxyproline-containing peptides, probably accelerated by bacterial collagenase.

The plateau of non-dialysable ^{14}C-hydroxyproline synthetized under sterile conditions, which is reached after

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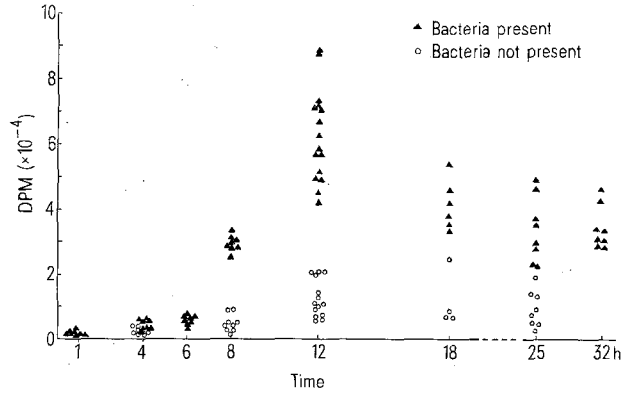

Fig. 2. DPM ^{14}C-hydroxyproline in intima-media of rabbit aorta per 2000 μg hydroxyproline as function of incubation time. △, Bacteria present; ●, Bacteria not present.

12 h of incubation, is in agreement with the results of JOUNI UITTO[1] and JUKKA UITTO[14]. The continuous progress in the incorporation of ^{14}C-proline was in accordance to the experiments of YEP et al.[15]. It is possible that protocollagen proline hydroxylase was inactivated before the incorporation of ^{14}C-proline was finished. The pH dependency of the incorporation and hydroxylation and the same optimal pH value was found earlier in studies of different connective tissues[1,14,11]. This indicates a similarity between the metabolic processes under study[16].

Zusammenfassung. Mit Bakterien im Inkubationsmedium wurde eine 10-bis 15-fache Erhöhung der ^{14}C-Prolin-Inkorporation und eine stimulierende Wirkung der ^{14}C-Hydroxyprolin-Synthese gefunden. Diese Beobachtungen zeigen, dass sterile Bedingungen notwendig sind, um falsche Schlussfolgerungen durch die Untersuchung des Kollagenmetabolismus mit ^{14}C-Prolin-Bildung in vitro zu vermeiden.

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Evidence for the Excretion of 2-Phenylethylamine Glucuronide in Human Urine[1]

The presence of 2-phenylethylamine (PEA), as a normal constituent of human urine, was first reported by JEPSON et al.[2]. Its levels are of medical interest because it is increased in phenylpyruvic oligophrenia[3] and in the manic phase of manic depressive psychosis[4], and decreased in patients with endogenous depression[5-7]. The identity of PEA in human urine[8], brain[9], and in peripheral organs[10] has been ascertained by us using a variety of procedures e.g., UV-, mass and IR- spectroscopy. Its occurrence in animals other than man has been reported by others and ourselves using UV-[11,12], mass and IR-spectroscopy[13] and by gas-liquid chromatographic procedures[14]. However, little is as yet known about the metabolism of PEA in vivo. More recently, BOULTON and MILWARD[6] found that in addition to free PEA, human urine contains conjugated forms of PEA: the nature of the conjugates is unknown. In this paper, we report evidence for suggesting that at least one form of the PEA conjugate excreted in human urine is β-PEA glucuronide.

Materials and methods. Morning urine sample (100 to 300 ml) from healthy, non-medicated human volunteers were collected into plastic vials containing 5 ml of a 1% solution of benzalkonium chloride, and used shortly after collection. Urine samples obtained from 10 subjects were pooled before experiment, and lots of 100 ml of urine were used in each determination.

After bringing the urine pH to 12.5 with 1 *N* NaOH, excess 15 ml 1 *N* NaOH were added to maximize the removal of amines other than PEA. Free PEA was extracted from the urine into 100 ml spectrograde n-hexane. The PEA in the organic solvent was then extracted into 10 ml 0.8 *N* HCl; 9 ml of this acid extract were used for the quantitative determination of PEA using the method of MOSNAIM and INWANG[10].

To diminish the possibility of urinary glucuronidase interfering with the assays, urinary enzymes were inactivated by incubating the urine samples at 65 °C for $^1/_2$ h in a water bath. The urine samples were then cooled to room temperature and filtered through Whatman No. 4 filter paper. The filtrate was divided into 3 aliquots to be used in the following experiments: a) extraction and determination of free PEA as described above, followed by acid hydrolysis (addition of concentration HCl and heating in a water bath at 100 °C for 30 min) of the conjugate substances remaining and subsequent extraction and determination of the PEA freed by this hydrolysis (conjugated PEA); b) acid hydrolysis (as described above) of the urine followed by extraction and determination of PEA (free and conjugated); c) extraction and determination of PEA from urine samples treated with β-glucuronidase as follows: 100 ml lots of the urine sample were brought to pH 5.3 with acetate buffer. 5 ml of a 5% solution of β-glucuronidase (50,000 units; K & K Labs.) in sodium acetate buffer were added, and the flask was incubated with shaking for 1 h in a water bath maintained at 37 °C. Enzyme activity was stopped by adding 10 ml of 2 *M* NH_4OH.

Results and discussion. As shown in the Table, total PEA (free and conjugated) obtained after acid hydrolysis (551 µg/l) was almost identical to the sum of the free and conjugated forms of PEA obtained separately (568 µg/l) and was also similar to the value obtained (515 µg/l) when β-glucuronidase was substituted for HCl in the hydrolysis, indicating that most urinary conjugated PEA exist as its β-glucuronide. The observed excretion rates for free PEA were comparable to those obtained by other investigators using a fluorometric method[4] (mean 336 µg/24 h; range 105–775) and gas chromatography[15] (mean 450 µg/24 h; range 116–1420). Lower values were

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