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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Experimental condition		PEA µg/l urine mean[a] ± S.E.
a)	Urine extracted without acid hydrolysis (free PEA)	292 ± 45
	Urine extracted after acid hydrolysis (conjugated PEA)	276 ± 62
b)	Urine extracted after acid hydrolysis (free + conjugated PEA)	551 ± 78
c)	Urine extracted after incubation with glucuronidase (free PEA + PEA glucuronide)	515 ± 69

[a] Average of 5 replicates.

reported using the PEA-alloxan fluorometric technique[6] (mean 47 µg/24 h, range 5–167). Values for urinary conjugated PEA levels are not available in the literature.

Recovery studies indicated that over 95% of the free PEA was removed from the urine after a single n-hexane extraction; a second extraction yielded less than 5% more free PEA. However, after the extraction and removal of free PEA from the urine, and the conjugated substances alone were subjected to either acid hydrolysis or incubated with β-glucuronidase under the conditions previously described, additional quantities of PEA were extractable in each case which accounted for approximately 50% to 60% of the total PEA (free plus conjugated). In most urine samples, the PEA values obtained by acid hydrolysis of the conjugated substances were similar to the values obtained by enzymatic hydrolysis. However, in some urine samples, significantly higher values of conjugated PEA were obtained by acid hydrolysis [69.8 ± 15.7 Mean (of 5 replicates) ± S.E.] than by β-glucuronidase degradation [36.0 ± 9.0 Mean (of 5 replicates) ± S.E.]. These results led us to suggest that PEA glucuronide is one of the major excretion products of conjugated PEA, but there may be other forms, probably ethereal sulfates or PEA associated with uric acid.

Zusammenfassung. Nachweis, dass die Ausscheidung von 2-Phenylaethylamin-Glucuronid im Urin von gesunden Versuchspersonen zwischen 50 und 60% der Gesamtmenge beträgt.

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On the Fractionation of Halophilic Enzymes with Ammonium Sulphate

The enzymes from extremely halophilic bacteria require high salt concentrations for both activity and stability[1,2]. The salt requirement for stability makes purification of these enzymes a difficult task, since very few methods can be applied successfully in the presence of NaCl concentrations ranging from 3 to 5 *M*. Ammonium sulphate fractionation, perhaps the commonest procedure in enzyme purification, is very difficult in the presence of high concentrations of monovalent cations[3]. Different ways of avoiding this difficulty have been reported in the literature, namely precipitation on the enzme after removal of the NaCl, followed by renaturation by dialysis against 5 *M* NaCl[3]; precipitation after removal of the salt, protecting the enzyme under study with substrates[4,5], and direct precipitation with solid ammonium sulphate starting with a crude extract in 2 *M* $(NH_4)_2SO_4$[6]. The first procedure usually gives low yields[3,4]; the second can be applied only to enzymes susceptible of protection by substrates or cofactors. The results presented here show that considerable purification, with reasonably good yields, of several halophilic enzymes, can easily be attained by direct fractionation of a crude extract in 5 *M* NaCl with a saturated solution of ammonium sulphate.

The strain of *Halobacterium cutirubrum*, the conditions of growth, harvesting and washing of the cells, the preparation of the cell-free extracts, the DNAase treatment, and the determinations of protein, were as previously described[7,8]. The crude extract, in 0.05 *M* *Tris*-HCl buffer (pH 7.6), containing 5 *M* NaCl and 1 m*M* EDTA, was brought successively to the following concentrations of ammonium sulphate: 2.6 *M*; 3.03 *M*; 3.25 *M*; 3.57 *M*; 3.68 *M*; 3.9 *M* and 4.1 *M*, by the addition of either saturated ammonium sulphate solution (containing 1 m*M* EDTA and adjusted to pH 7.0 with NH_4OH) (fractions P_1 to P_5) or solid ammonium sulphate (Fractions P_6 and P_7). The concentration of the saturated ammonium sulphate solution was taken as 3.9[9], since the fractionations (with the exception of the last one) were performed at 0–5 °C. The concentrations listed above correspond, therefore, to 66; 77.6; 83.3; 92; 94.3; 100% (at 0 °C) and 100% (at 25 °C) saturation respectively. In all cases the addition of the ammonium sulphate was made slowly and with efficient stirring. After an additional 10 min stirring period, the suspensions were centrifuged for 20 min at 37,000 × *g*. The bulky red precipitates obtained from the first and second fractions were washed with an $(NH_4)_2SO_4$-NaCl solution of the appropriate concentration, centrifuged again, and the washings pooled with the original supernatant fluids. All precipitates were redissolved in 1 ml of the *Tris*-HCl- 5 *M* NaCl- EDTA solution and used as such for the enzyme assays. In some of the experiments the saturated ammonium sulphate solution contained 0.3 m*M* NADH. For the experiments with solid ammonium sulphate, the cell-free extract was dialysed overnight against 100 volumes of 0.05 *M* *Tris*-HCl buffer (pH 7.6) containing 2 *M* $(NH_4)_2SO_4$ and 1 m*M* EDTA, and then the dialysed extract was brought to the same ammonium

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