position, and it is not given by pyridones. Hence the hydroxyl group can only be in the 3-position of the pyridine ring or in the benzene ring ortho or meta to the primary amine. Through the courtesy of Dr. A. R. Day, who prepared the ortho hydroxy derivative of sulfapyridine, we have been able to rule out this possibility. The meta hydroxy derivative has not been ruled out, but we have been inclined to favor the 3-position in the pyridine ring as being the more plausible. This postulation is in agreement with the recent work of Weber *et al.*[7] which definitely places the hydroxyl group in the pyridine ring.

Discussion. These results indicate that the urinary elimination of sulfapyridine is more complex than hitherto recognized. Since the hydroxysulfapyridine and the water-soluble glucuronide possess free arylamino groups they are measured by diazotization procedures along with the "free" sulfapyridine. Their therapeutic efficiency may not be equivalent to that of the unchanged drug. Further, the excretion of a part of the drug in a highly soluble form is important in the etiology of sulfapyridine urolithiasis.[8]

Summary. Following oral administration of sulfapyridine, a hydroxysulfapyridine and a water-soluble hydroxysulfapyridine glucuronide have been isolated from dog urine.

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[8] Scudi, J. V., and Robinson, H. J., *Am. J. Med. Sci.*, 1941, **201**, 711.

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Microbiological Differentiation of Pyridoxine and Pseudopyridoxine.*

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Snell, Guirard, and Williams[1] obtained abnormally high pyridoxine values when urine was assayed with *Streptococcus lactis R* against a pyridoxine standard. These workers demonstrated that the effect was due to the presence in urine of a metabolite of pyridoxine which they called pseudopyridoxine. It was found that pseudopyridoxine could be formed by the interaction of pyridoxine and amino acids during autoclaving.[2]

Using yeasts and molds for pyridoxine assay, other workers (Robbins and Ma,[3] Carpenter *et al.*,[4] Atkin *et al.*,[5] and Stokes *et al.*,[6]) did not obtain abnormally high values on materials known to contain pseudopyridoxine. It has therefore been postulated that these organisms do not respond to pseudopyridoxine.

While Snell *et al.*[2] obtained evidence that *S. lactis* utilizes only pseudopyridoxine and not pyridoxine, no conclusive evidence has been obtained that yeasts do not utilize both pyridoxine and pseudopyridoxine. The high assay values obtained with *S. lactis* merely reflect the small conversion of pyridoxine to pseudopyridoxine in the standard. If both compounds are active for an organism, abnormally high assay values would not be expected.

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

[1] Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

[2] Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 356; *J. Bact.*, 1943, **45**, 199.

[3] Robbins, W. J., and Ma, R., *Proc. Nat. Acad. Sci., U. S.*, 1942, **29**, 172.

[4] Carpenter, L. E., Elvehjem, C. A., and Strong, F. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 123.

[5] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

[6] Stokes, J. L., Larsen, A., Woodward, C. R., Jr., and Foster, J. W., *J. Biol. Chem.*, 1943, **150**, 17.

In order to obtain specific evidence as to whether yeasts can utilize pseudopyridoxine, we have made the two studies reported below.

Experimental. The following work was carried out on human sweat which was found to contain pseudopyridoxine as well as pyridoxine.

The method used for the separation of pyridoxine and pseudopyridoxine in sweat involved the elimination of pyridoxine by yeast growth, filtration of yeast cells, and assay of the filtrate for pseudopyridoxine. In a similar manner pseudopyridoxine could be eliminated by bacterial growth, the bacterial cells removed by filtration, and the filtrate assayed for pyridoxine.

A sample of pooled filtered sweat, concentrated 2 to 1, contained 0.0018 μg of pyridoxine per cc by the *Saccharomyces carlsbergenesis* procedure of Atkin, Schultz, Williams, and Frey,[5] and 4 μg of pyridoxine equivalents of pseudopyridoxine using the *Lactobacillus casei* procedure of Carpenter, Elvehjem, and Strong.[4]

Four cc of this filtered sweat and 1 cc of *Sacch. carlsbergensis* inoculum were added to 5 cc of the medium of Atkin *et al.*[5] Less than maximum growth of the yeast was obtained in 24 hours. The yeast cells were filtered off and the filtrate assayed with *L. casei.* Four μg per cc of sweat of pyridoxine equivalent of pseudopyridoxine were found in the filtrate. The same medium after filtering off the yeast cells was reinoculated with yeast and no growth was obtained, indicating that the pyridoxine had been completely utilized by the first yeast growth. When 5 and 20 mμg of pyridoxine were added to the filtrate 99% was recovered, showing that growth had not been prevented by the presence of any inhibitory substances which might have been formed by the original yeast growth.

L. casei was grown on the medium of Landy and Dicken,[7] omitting the pyridoxine, and the same sweat as before was added at 10 levels from 0.1 to 5 cc per tube. After 72 hours the cells were removed by filtration. The pyridoxine was recovered from the filtrate by adsorption and elution from Lloyd's reagent and the amount found by yeast assay was 0.0016 μg per cc of sweat. This value corresponds closely to the value of 0.0018 μg per cc obtained on the original sweat sample. Another aliquot of the casei filtrate was re-inoculated with *L. casei* and no growth was obtained. When pseudopyridoxine† was added, growth of *L. casei* was obtained.

These experiments demonstrate that yeast can use pyridoxine and not pseudopyridoxine, whereas *L. casei* uses only pseudopyridoxine and not pyridoxine.

The assay values on sweat obtained by the yeast *Sacch. carlsbergensis* correspond with those obtained by *Sacch. oviformis*[8] and the mutant mold *Neurospora sitophila* 299.[6] Apparently therefore, these organisms also respond only to pyridoxine. In addition to *L. casei*[4] and *S. lactis R*,[1,2] *Leuconostoc mesenteroides* was also found to respond to pseudopyridoxine and therefore cannot be used as an assay organism for pyridoxine as suggested by Gaines.[9]

Since *L. casei* uses only pseudopyridoxine, it could be used for the assay of this compound if pseudopyridoxine were available for a standard. An assay using pyridoxine, itself, as a standard is not accurate due to the variability of the conversion of pyridoxine to pseudopyridoxine during sterilization. This fact was shown by assaying sweat samples for pseudopyridoxine with *L. casei* using three different sterilization times, 10, 15, and 20 minutes. The assay values obtained were respectively, 10, 4, and 0.13 μg per cc of pyridoxine equivalent of pseudopyridoxine. The assay values decreased as more of the pyridoxine in the standard was converted to pseudopyridoxine.

Work was carried out on the separation of pyridoxine and pseudopyridoxine without recourse to micröorganisms and some success was obtained with Lloyd's reagent.

Fifty cc of a pooled sample of sweat were

† By adding pyridoxine in large amounts, 0.1 to 1.0 μg per tube before sterilization. Amounts of pyridoxine less than 0.1 γ/tube do not give any stimulation of *L. casei* at the sterilization times used here (up to 20 minutes).

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TABLE I.
Separation of Pyridoxine and Pseudopyridoxine by Means of Lloyd's Reagent.

	Filtrate, γ/cc	Eluate, γ/cc
Pyridoxine *Sacch. carlsbergensis*	0.0000	0.0016
Pseudopyridoxine *L. casei*	0.16 equivalents	0.31 equivalents

brought to pH 3.0 with sulfuric acid. 0.5 g of Lloyd's reagent was added to absorb the pyridoxine. The pyridoxine was eluted with 15 cc (three 5-cc portions) of 1/10 N NaOH. This eluate was neutralized and diluted to 25 cc. Both the solution left after the Lloyd's reagent absorption (concentrated to 25 cc) and the eluate were analyzed for pyridoxine and for pseudopyridoxine. The results are given in Table I.

From this table it can be seen that while about two-thirds of the pseudopyridoxine follows the pyridoxine in its absorption as found by Snell *et al.*,[1] the filtrate does contain considerable pseudopyridoxine and no pyridoxine. Thus some separation was secured and again the yeast was unable to utilize the pseudopyridoxine.

Summary. Pyridoxine has been separated from pseudopyridoxine by using the yeast *Saccharomyces carlsbergensis* and the bacterium *Lactobacillus casei*. It has been shown that *Sacch. carlsbergensis* assays and utilizes only pyridoxine while *L. casei* assays and utilizes only pseudopyridoxine.

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Ovarian Tumors in Adult Rats Following Prepuberal Administration of Estrogens.*

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Estrogens injected into lactating rats have been shown to be transmitted to the suckling young inducing changes in urogenital organs of both sexes.[1] The effects are of such a nature that it seemed advisable to make further observations on similar animals, at intervals after cessation of treatment, in order to determine the permanency of the condition and the course of any subsequent changes. It has been reported that female rats given prepuberal injections of androgen[2-4,6] or estrogen[5-7] have severe reproductive deficiencies as adults. Because of variations in methods of administration, hormones used, and length of treatment, the present study brings out differences thought to be of significance.

Methods. We have observed that in untreated female littermates of immature animals given daily injections of diethylstilbestrol, the vaginas open at the same time as in injected animals. This has been confirmed repeatedly with large litters containing only one injected animal. Similar changes have been observed in litters confined to cages in which stilbestrol was sprayed upon shavings used as nesting material. Injection of cagemates or contamination of nesting material with stilbestrol may therefore be used as alternative methods of administering hormone to immature animals.

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