Lewis and Wright(7) expressed the view that the mouse ovum is always fertilized before loss of its follicle cells, and Leonard, Perlman, and Kurzrok(1), as well as Austin(2) demonstrated that the recently fertilized rat ovum is usually found still enclosed by these cells.

In these previous experiments, however, no attempt was made to determine where fertilization occurred. There exists the possibility that as the ovum is forced from the ovary the associated follicle cells might be pulled aside sufficiently to facilitate entrance of the awaiting sperm. A subsequent closing-in of these cells would then give the impression that the sperm had worked its way through the entire surrounding follicle cell mass. In the present experiment, however, it was known that the ova surrounded by their follicle cells were in the ampulla of the oviduct before arrival of the spermatozoa, and yet the sperm were able to effect fertilization.

It will be noted in Table I that the first 9 animals yielded 24 fertilized ova out of a possible 47 recovered, whereas of the remaining animals, only 2 possessed ova with sperm. These first 9 animals were all bred before sunrise, but due to progression of the spring season the remaining rats were bred after sunrise. It is possible that with the approach of longer days, the ovulation time was shifted to an earlier period with the result that the inseminations may have taken place too long after ovulation for fertilization to succeed. It is true that the lapsed time between insemination and examination of the ova for sperm was somewhat shorter in the animals bred after sunrise, but one rat had ova fertilized by 4 hours post-coitus. It could not be for the lack of sperm that fertilization failed in these animals since spermatozoa were observed in all of the oviducts but one. Blandau and Money(11) have reported spermatozoa in the ovarian portion of 100% of rat oviducts examined one hour after copulation.

Summary. Rats were bred late in estrus and the presence of ova in the oviducts determined by immediate removal and examination of one of the ducts. By later removal and examination of ova from the other oviduct, it was possible to demonstrate that rat sperm could fertilize non-denuded ova within the oviduct. The incidence of fertilization was markedly lowered by copulation late in estrus.

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Determination of Urea in Saliva. (18412)

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Although the concentration of urea plus ammonia (U + A) in saliva is known on the average to approximate that in blood(1-5), the determination of U + A in saliva has not come into clinical use, probably on account of the wide variations encountered in individual samples.

Various methods for blood urea have been applied to saliva. Whatever the chemical technic used, the manner of collection of saliva samples is more important. In most previous studies, the volume of saliva required for analysis was relatively large, and, to obtain sufficient samples, artificial stimulation of the salivary glands by chewing paraffin was resorted to. The increased rate of flow due to

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4. Pacetto, G., *J. Am. Med. Assn.*, 1925, v85, 934.

5. Centeno, A. M., *J. Am. Med. Assn.*, 1931, v97, 745.

6. Barnett, G. D., and Bramkamp, R. G., PROC. SOC. EXP. BIOL. AND MED., 1929, v27, 118.

stimulation tends to lower the salivary U + A, as Barnett and Bramkamp(6,7) have shown. On the other hand, slow-flowing saliva obtained without stimulation may give U + A values which are even higher than that of blood urea. This is probably due to contamination by products of bacterial action on food debris or on nitrogenous constituents, other than urea, in saliva itself(8). The contamination is greater when the time required for collection is prolonged.

Using Conway's micro-diffusion technic(9, 10) which has not been applied to saliva before, U + A can be determined with 1 ml or less of saliva. Sufficient saliva for duplicate analyses can be collected in a few minutes without artificial stimulation, and the chief cause of variation of the U + A value, as mentioned above, is thus removed.

Experimental. Collection of sample. Half an hour before the collection of a sample the teeth are brushed or the mouth thoroughly rinsed. The saliva is then swallowed continually for about 15 minutes to remove the remnant of the rinse water. One minute before the time of collection, the edge of a shortstem funnel (50 mm diameter) is pressed lightly against the lips. With mouth moderately open and head leaned forward, saliva is allowed to flow freely into a 10 ml graduate until about 2.5 ml have been collected. This requires only a few minutes. The moment when saliva actually begins to flow into the funnel is taken as the initial time of the collection. No sucking or spitting is necessary and should be avoided. Saliva collected in this way is usually water clear. During the collection of the sample, care is taken not to breathe through the mouth to avoid dilution of the sample by condensed moisture.

Determination of U + A. The determination of U + A is carried out in a 68 mm diffusion unit according to Conway. The procedure of Steinitz(11) was followed with slight modifications. Into the central compartment of the diffusion dish is measured 1 ml of a 2% boric acid solution containing an appropriate amount of methyl red + methylene blue as indicator. Into the outer compartment is delivered 1 ml of saliva and 0.5 ml of urease solution. The urease solution is prepared by dissolving eight urease tablets* in 10 ml water and adding 0.3 ml of a phosphate buffer solution (6.9 g of $NaH_2PO_4 \cdot H_2O$ and 17.9 g of $Na_2HPO_4 \cdot 12H_2O$ in 100 ml water). The urease is allowed to act on saliva for 10 minutes before adding 1 ml of saturated potassium carbonate. After 90 minutes the ammonia absorbed in the 2% boric acid solution is titrated with N/70 HCl from a 1 ml micro-burette.† For 1 ml of saliva, the amount of HCl required for titration ranged from 0.4 to 1.0 ml. Duplicate determinations usually agree within 0.01 ml of acid. The ammonia-N thus determined, after correction for blank, represents U + A nitrogen. The salivary ammonia-N which can be separately determined by the same procedure by replacing the urease with 0.5 ml of ammonia-free water usually amounts to 1-2 mg of N per 100 ml saliva. As little as 0.2 ml of saliva suffices for a determination. To minimize experimental errors, we chose to use 1 ml in our present study.

Results and discussion. The reproducibility of salivary U + A values is shown by experiments in which saliva samples were collected at frequent intervals. In one subject, samples collected at 10 minute intervals from 9:30 A.M. to 11:00 A.M. gave values ranging from 10 to 11.2 with an average of 10.6 ± 0.4 (S.D.) mg N per 100 ml of saliva. In another subject, samples taken every 15 minutes from 8:50 A.M. to 10:50 A.M. gave values ranging from 9.2 to 10.2 with an average of 9.7 ± 0.3 (S.D.) mg N per 100 ml saliva.

Table I shows some salivary U + A values

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11. Steinitz, K., *J. Lab. and Clin. Med.*, 1939-40, v25, 288.

* Made by Hynson, Westcott and Dunning, Inc., Baltimore. Each tablet contains 25 mg urease.

† This can be improvised by drawing the tip of a 1 ml graduated pipette to a capillary and attaching a 2 ml syringe to the upper end.

TABLE I. Salivary Urea in Normal Persons. Mg urea plus ammonia nitrogen per 100 ml saliva.

Subject, sex and age	Date	Time of collection										Approx. rate of flow, ml/hr
		6	8	9	10	11 A.M.	12 N.	1	2	3	4 P.M.	
H, M, 56	May 5				12.6		13.0		12.8		11.6	45
	" 6	12.8	12.6		10.8		12.8					
	" 11				11.1	15.1	11.9	15.0	14.6	13.9		
	" 12	11.5	10.9	12.8	11.7	11.7	12.2	10.1	10.9	12.6		
	Oct. 24		11.4	12.1	12.6	12.8	11.5	12.0	12.3	11.5		
Y, F, 48	June 7					12.4	14.3			15.3		25
	" 14			16.7	14.7	15.7	16.1			17.3	16.7	
	Oct. 25			10.3	10.4	12.1	11.4	11.3	10.1	10.4	10.4	
J, M, 32	June 16			17.6	18.1	15.0	15.9		16.4	18.6		30
	Oct. 23			13.1	14.5	11.9	12.8		11.8	13.6		
G, M, 30	June 22			11.4	9.1	8.7	9.0	8.8	11.3	9.5		30
S, M, 28	" 21				15.6	11.3	13.4	10.8	13.3	13.8		15
D, F, 22	" 8				9.9	11.1	11.2		10.3	11.7		12
	" 15			15.5	14.5	12.5	12.5		12.2	12.8		
R, M, 21	" 8				7.4	8.2	8.6		8.2	9.7		25
C, F, 20	" 9			11.3	11.1	11.3	11.3		9.8	11.0	10.2	20
V, M, 18	" 7					9.7	10.4		9.4	9.4		15
	" 9			9.2	11.1	11.7	9.9		8.4	9.7	10.4	

in normal adults. It will be noted that in some persons the U + A concentration is practically constant from hour to hour. In others, there is some variation which may reflect a variation of the urea concentration of the extracellular fluid after intake of food or drink. While the average level of U + A in the same person may change considerably from season to season (*e.g.* subjects J and Y), it remains the same from day to day in the same season (*e.g.* subjects, H, D, and V).

Whether the salivary U + A is exactly equal to blood urea or not, it has a significance of its own. Since the determination of U + A in saliva by the micro-diffusion method is so simple, it deserves consideration as a routine test in the clinical laboratory.

Becks and Wainwright(12) called attention to the fact that human saliva is a secretory and or excretory product and that the rate of secretion of a substance as well as the concentration of the substance in saliva should be considered. Morris and Jersey(13) reported that the rate of urea secretion of the resting gland was more uniform than the concentration of urea. But this conclusion was not borne out by their data (Table III in their paper).

Since the calculation of the rate of salivary urea secretion requires a knowledge of the rate of flow which is much more variable than urea concentration and which cannot be accurately determined in short intervals, it seems better to express salivary U + A values simply in terms of concentration and not as rate of secretion.

Summary. Using Conway's micro-diffusion technic, urea plus ammonia determinations can be made with 1 ml or less of saliva. Sufficient saliva for duplicate analyses can be collected in a few minutes without artificial stimulation. Saliva collected in this way gives reproducible values in the same individual.

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13. Morris, J. L., and Jersey, V., *J. Biol. Chem.*, 1923, v56, 31.

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