day. On these bases, the agent was considered to be the virus of eastern equine encephalomyelitis.

To our knowledge, this is the first report of the isolation of the eastern variety of equine encephalomyelitis virus from the blood of a naturally infected wild bird. It is of added interest that the specimen was collected during a period when there were comparatively few cases of the disease reported in horses in the region.

Summary. The virus of eastern equine encephalomyelitis was isolated from the blood of an adult purple grackle collected in Louisiana in June, 1950.

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A Rapid Assay for Xanthurenic Acid in Urine. (18792)

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In man(1) and other mammalian species (2-6), a deficiency of pyridoxine has been shown so to alter the normal metabolism of tryptophan as to result in the urinary excretion of xanthurenic acid (XA). Following the ingestion of tryptophan, the quantitative measurement of XA in urine has been used as an index of pyridoxine deficiency or dysfunction(1). Colorimetric methods available at present for the determination of XA in urine generally involve preliminary time-consuming extraction of XA from urine prior to colorimetric assay(3,7,8).

In view of the increasing significance of pyridoxine in disorders of pregnancy(9-11) and possibly other clinical states(12-14), a rapid, reliable method for the estimation of XA in human urine would be of definite advantage for clinical studies where a large number of routine assays are to be performed. It is the purpose of this paper to present such a method.

Reagents and apparatus. 1. Five % sodium bicarbonate solution, freshly prepared prior to analysis. 2. $FeNH_4(SO_4)_2 \cdot 12H_2O$ solution. 0.85 g of Mallinckrodt analytical reagent grade, dissolved in 50 ml water. 3. Sodium hydrosulfite powder (J. T. Baker Chemical Co.). 4. Standard xanthurenic acid* solution: Stock standard solution, 500 μg per ml of XA, made by dissolving 25 mg of XA in 50 ml of 5% ethyl alcohol which contained 0.1 ml of concentrated ammonium hydroxide.

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* The xanthurenic acid used in these studies was synthesized in our Division of Organic Chemistry by Mr. A. Mebane and Dr. W. Oroshnik. This substance has a melting point of 297-298°C. An elementary analysis revealed the following: $C_{10}H_7NO_4$: Calculated. C, 58.54; H, 3.44; N, 6.83. Found. C. 58.40; H, 3.65; N, 6.66; Ash, 0.

This solution was stable for at least one month when kept under toluene in the refrigerator. The working standard solution, 100 μg per ml, was made by diluting 4 ml of the stock standard to 20 ml with distilled water. This solution should be prepared daily. 5. The Evelyn colorimeter was used with a 620 mμ filter.

Procedure. The urine was adjusted between pH 7.3-7.5 and then filtered through No. 1 Whatman filter paper. When XA assays were performed on a urine specimen following the administration of a test dose of tryptophan(1) the urine was diluted with water to contain 25-100 μg of XA per ml; it was found unnecessary to dilute specimens obtained before the ingestion of tryptophan. From 1 to 4 ml of urine (undiluted if small quantities of XA were present) were measured into each of two calibrated Evelyn tubes. To one, there was added 1 ml of the working standard containing 100 μg of XA to determine the XA recovery value; to the other tube (the actual test specimen) no standard XA was added. The contents of both tubes were diluted to 9 ml with water. To each tube, there was added 10 to 15 mg of sodium hydrosulfite, contained on the tip of a spatula, and this was followed by 0.10 ml of 1.7% $FeNH_4(SO_4)_2 \cdot 12H_2O$. It is important that the addition of the hydrosulfite precede the addition of the ferric ion in order to prevent the formation and subsequent precipitation of ferric hydroxide. The contents of the tube were then thoroughly mixed by gentle agitation. One ml of 5% sodium bicarbonate solution was now added to each tube. After gentle mixing so as to prevent the oxidation of the hydrosulfite by air, the blank values of both tubes were determined as described below. The colorimeter was first adjusted by setting the galvanometer at 100 with an aqueous solution containing 100 μg of pure XA per 10 ml assay tube which served as the reagent blank. This solution was prepared with the same quantities of reagents employed in the urine tubes above, except that 0.025 ml in place of 0.10 ml of 1.7% $FeNH_4(SO_4)_2 \cdot 12H_2O$ was added to the tube. The galvanometer readings of the blank tubes, *i.e.* the color of the urine and reagents in the reducing medium were then determined in the photoelectric colorimeter. After obtaining these readings, the contents of each tube were shaken well to permit oxidation until the green ferric-XA complex salt was formed and remained stable. Galvanometer readings were again made. All galvanometer readings were then converted to "L" values (Evelyn), and calculations were carried out in the conventional manner. Several points are worthy of note. The color was found to fade rapidly if the hydrosulfite was not completely oxidized. On the other hand, the color was stable for a period as long as 3 hours if destruction of the reducing agent was complete. When pure XA was analyzed, the excess iron slowly precipitated as the hydroxide. This resulted in a gradual loss of color. Thus it was necessary to read solutions of pure XA within 10 minutes after color was developed. Such precipitation did not occur in urine.

Results. The values obtained for urine and for standard solutions of XA were reproducible on different days. The color obtained obeyed Beer's law for solutions ranging from 10-250 μg of XA; a calibration curve was required when larger amounts of XA were measured.

The reliability of the test was examined by determining recovery values of XA added to urine specimens containing varying quantities of inherent XA. Thus at one extreme normal non-pregnant women have been shown to excrete very little urinary XA after a test dose of tryptophan; pregnant women close to term in certain pathognomonic states and preeclamptic women excrete large quantities of urinary XA under the same conditions(15). Typical data are presented in Table I.

Effect of time, temperature and pH. In the urine assay, the color reached a maximum immediately after oxidation of the hydrosulfite and remained stable in the daylight for at least 3 hours at room temperature. The color of the complex salt was stable between 15 and 37°C. The color intensity of the ferric-XA compound was at a maximum between

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TABLE I. Recovery of Xanthurenic Acid Added to Urine.

Urine No.	Amt urine assayed, ml	Urinary XA, μg/ml	Added XA, μg	XA recovered, μg	% recovery
1	4	3	0		
1	4		25	19	76*
1	4		50	40	80*
1	4		100	100	100
1	2	2	0		
1	2		25	26	104
1	2		50	49	98
1	2		100	92	92
2	1	126	0		
2	1		10	11	110
2	1		25	30	120
2	1		50	58	116
3	.2	457	0		
3	.2		100	109	109
4	.2	316	0		
4	.2		100	110	110

* When small quantities of XA are recovered in presence of large volumes of urine, recovery values are apt to be low because of interfering urinary pigments.

pH 7.0 and 8.2; it dropped off sharply on either side of this optimum range. It was essential, therefore, that before analysis the urine be adjusted between pH 7.3 and 7.5. Finally, the assay solution was buffered with bicarbonate, since an excess of hydrosulfite (which was not added quantitatively) brought the pH below 7.0.

Choice of buffer. Attempts to utilize more efficient buffers such as potassium bicarbonate and disodium phosphate in place of sodium bicarbonate were ineffective; the phosphate buffer gave rise to the formation of the insoluble ferric phosphate at pH 7.8. A 25% solution of potassium bicarbonate at room temperature lost carbon dioxide rapidly; this resulted in the formation of the highly alkaline potassium carbonate.

Specificity of reducing agents. The choice of reducing agent was found to be restricted to sodium hydrosulfite. Attempts to use other reducing agents such as ascorbic acid, sodium sulfite, and sodium bisulfite were unsuccessful. Compared to sodium hydrosulfite, the lower reducing potentials of these substances would explain these findings. It should be noted that excessive quantities (greater than 20 mg) of sodium hydrosulfite cannot be used in this procedure, for under such conditions the acidity of the assay solution might be so increased as to prevent the return to the desired pH range 7.0-8.2 upon the addition of sodium bicarbonate.

Discussion. It could be demonstrated by paper chromatographic methods and absorption spectrum studies(16) that the green color resulting on the addition of ferric ion to urine was due *specifically* to XA. Although the exact chemical nature of the Fe-XA complex remains yet to be determined, the amount of ferric ion used in this assay was well beyond the critical concentration necessary for optimum color development. This was established as follows: When a given amount of XA was titrated with increasing amounts of ferric ion, the maximum color intensity occurred in a ratio of one mole of ferric ion to 2 moles of XA. In the procedure described herein, approximately 10 times this amount of ferric ion was added to all tubes containing urine. This was necessary since urine is known to contain chelating substances which combine with iron and thereby render it unavailable for coordination with XA.

Summary. A colorimetric method for the direct estimation of xanthurenic acid in urine without preliminary extraction is described. The method is based on the reduction of the ferric to ferrous ion by sodium hydrosulfite to obtain a reliable blank value and the subsequent oxidation of the reactants to obtain the green ferric-xanthurenic acid complex salt. Provisions are made for the use of an internal recovery standard with each sample of urine assayed.

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