

# MAPLE SYRUP URINE DISEASE

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A familial syndrome characterized by gross mental retardation, a urine odour resembling maple syrup, and a short fatal course, was first described by Menkes, Hurst and Craig (1954). Two similar cases have since been reported (Westall, Dancis and Miller, 1957; Dancis, Levitz, Miller and Westall, 1959; Mackenzie and Woolf, 1959), in which markedly raised levels of leucine, isoleucine and valine were found in urine and plasma. Analysis also revealed high concentrations of the  $\alpha$ -keto acids derived from these branched-chain amino acids by transamination (Menkes, 1959).

These abnormalities could result from a defect in the further degradation of the keto acids, for which the most probable normal pathway involves a common mechanism of oxidative decarboxylation (Coon, Robinson and Bachhawat, 1955; Fig. 1), and recent work has sought to substantiate this inference.

A study of one of the cases mentioned above has been summarized recently by Dancis, Levitz and Westall (1960). The further biochemical investigation of the other case was undertaken at this hospital, and the results obtained are presented in this report.

## Case History

A full report on first admission of the child to The Hospital for Sick Children has been given by Mackenzie and Woolf (1959). Dietary trials carried out at University College Hospital are the subject of the previous paper in this issue (Dent and Westall, 1961).

The patient, a girl, was the only child of healthy, unrelated parents, with no family history of mental deficiency. She appeared normal at birth, but stopped sucking at 1 week, and had to be fed by tube.

On admission to The Hospital for Sick Children, aged 4 months, she was severely mentally retarded, and some jerking of the limbs was noted. The urine possessed a persistent odour resembling burnt sugar, and analyses of urine and plasma for amino and keto acids confirmed the suspected diagnosis of maple syrup urine disease. A diet containing glycine as the sole amino acid was tried, but no improvement in the clinical or biochemical findings was observed. When 8 months old she was transferred to the metabolic unit of University College

Hospital, for further investigation of restricted diets, and from there was admitted to Botley's Park Hospital, where she died on August 23, 1959, aged 15 months. The post-mortem findings are to be published by Crome, Dutton and Ross (1961).

## Materials and Methods

A 24-hour collection of urine from the patient at 8 months of age had been stored at  $-25^{\circ}$  C. for nine months. Post-mortem specimens of liver, kidney and brain were frozen immediately and kept at  $-25^{\circ}$  C. Control specimens from a patient of about the same age, who died from congenital heart disease, were treated in the same manner.

**Chromatography.** Amino acid chromatography was carried out in the usual way on Whatman No. 1 paper, using n-butanol-acetic acid-water (12:3:5 by vol.) as solvent system. Tissue incubation media were deproteinized with ethanol, and concentrated to small volume for analysis. These solutions were analysed also for metabolic intermediary acids (Lugg and Overell, 1948). For the detection of coenzyme A derivatives (Stadtman, 1952), media were deproteinized by an equal volume of ethanol adjusted to pH 4.5 with 0.1 N sodium acetate buffer.

Urinary keto acids were characterized as the 2:4-dinitrophenylhydrazones in a butanol-ethanol-ammonia solvent (El Hawary and Thompson, 1953). Trichloroacetic acid filtrates of tissue media were analysed similarly. The  $\alpha$ -ketoisocaproic acid used as a standard was obtained from the California Corporation for Biochemical Research, Los Angeles, and purified by recrystallization of the barium salt from aqueous acetone.

An ether-soluble fraction of acidified urine was obtained by continuous extraction for 12 hours. After removal of ether, the residual oil was dissolved in dilute ammonia, and chromatographed on Whatman No. 2 paper, with a solvent system, n-butanol-n-propanol-0.1 N ammonia (2:1:1 by vol.). Ammonium salts produced red spots when sprayed with diazotized p-nitroaniline and alkali (Whitfield, 1960). Further information on the identity of the spots was obtained by using 2:4-dinitrophenylhydrazine and ferric chloride-phenol as spray reagents.

**Identification of  $\alpha$ -Hydroxy Acids.** Some conversion of urinary  $\alpha$ -hydroxy acids to the corresponding keto

acids was obtained by careful oxidation with Fenton's reagent. Urine (2 ml.) was diluted to 5 ml. with water, and cooled to 0° C. Ferrous sulphate solution (0.1 ml. of 0.001 M) and hydrogen peroxide (0.1 ml. of 0.2%) were added, and the mixture kept at 0° C. for six hours. While still cold, 0.4 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N HCl was added, and the mixture allowed to warm to room temperature. Extraction and chromatography of the resulting hydrazones was carried out as before.

Areas of paper occupied by individual spots were cut from a preparative chromatogram, and disintegrated in dilute acetic acid. The amino acids derived from these hydrazones by electrolytic reduction (Smith, 1958) were then identified by chromatography.

**Tissue Experiments.** Tissue homogenates were prepared in the appropriate ice-cold solutions, using an all-glass homogenizer of the Potter type.

**Transamination.** Incubation mixtures contained L-leucine (0.01 M), pyruvate or  $\alpha$ -ketoglutarate as the sodium salts (0.01 M), and 4 ml. of a 10% tissue homogenate in 0.1 M phosphate buffer, pH 7.4, in a total volume of 10 ml. The tests were run anaerobically at 37° C. Transamination was followed by periodic analysis for amino and keto acids.

**Oxidative Decarboxylation.** Tests for the anaerobic production of carbon dioxide were carried out by the standard Warburg method, using a medium of the following composition (concentrations expressed in  $\mu$ moles): potassium phosphate, pH 7.4, 200; magnesium sulphate, 2.5; cysteine, 10; diphosphopyridine nucleotide (DPN), 0.2; keto acid, K salt, 50; coenzyme A, 10; thiamine diphosphate, 0.2; adenosine diphosphate (ADP), 10. Tissue slices, or a 10% homogenate in 0.05 M phosphate, pH 7.4 (1 ml.), were used in a final volume of 2 ml. Incubation temperature was 37° C.

### Results

The characteristically raised levels of leucine, isoleucine and valine, and of the corresponding keto acids were the most prominent features of urine analysis. The 24-hour excretions of leucine and  $\alpha$ -ketoisocaproate were approximately 25 mg. and 65 mg., respectively. The keto acid pattern also showed an abnormally high level of  $\alpha$ -ketoglutarate.

The presence of  $\alpha$ -hydroxy acids in the urine was indicated by comparison of its reaction with 2:4-dinitrophenylhydrazine before and after oxidation with Fenton's reagent. The reaction was greatly increased after oxidation, the component showing greatest change ( $R_F$  0.82) coinciding with the dinitrophenylhydrazone of  $\alpha$ -ketoisocaproic acid. This identity, and the presence of the superimposed hydrazone of  $\alpha$ -keto- $\beta$ -methylvaleric acid, was confirmed by the formation of increased amounts of leucine and isoleucine by electrolytic reduction.

From these results it may be concluded that the urine contained  $\alpha$ -hydroxy-isocaproic and  $\alpha$ -hydroxy- $\beta$ -methylvaleric acids, the derivatives of leucine and isoleucine, respectively. The acid corresponding to valine, namely,  $\alpha$ -hydroxy-isovaleric acid, could not be detected by these methods.

Further confirmation of the identity of the urinary acids was derived from chromatography of the ether extractives. Two main components were coloured red by the diazo reagent, and each could be further resolved into two by the use of alternative spray reagents. The faster-moving areas ( $R_F$  0.50 and 0.38) reacted strongly with dinitrophenylhydrazine, and coincided with  $\alpha$ -ketoisocaproic and  $\alpha$ -ketoisovaleric acids, respectively. The slower-moving areas ( $R_F$  0.46 and 0.35) reacted for  $\alpha$ -hydroxy acids in giving a yellow colour with a phenol-ferric chloride spray, and coincided with  $\alpha$ -hydroxy-isocaproic and  $\alpha$ -hydroxy-isovaleric acids, respectively.

The oil derived from the ether extract also possessed much of the maple syrup odour, and this was found to be concentrated in two spots ( $R_F$  0.56 and 0.42) on the chromatograms. These constituents were completely separated from the above-mentioned acids, and were stained yellow by the diazo reagent, thereby differing from carboxylic compounds. Immediate reaction also occurred with dinitrophenylhydrazine and with ammoniacal silver nitrate. Some separation of the 'maple syrup substances' was obtained by gently warming the oil, to which a few drops of water had been added. Colourless needle crystals were deposited rapidly in the neck of the flask, and were shown to contain the 'maple syrup substance' of  $R_F$  0.56, together with some  $\alpha$ -hydroxy-isovaleric acid, by chromatography of a solution in dilute ammonia. The crystals were extremely volatile, disappearing in a few minutes on exposure to the air at room temperature.

**Enzyme Experiments.** The rates of transamination in a leucine-pyruvate system, as measured by the formation of  $\alpha$ -ketoisocaproate, were approximately the same for tissues of the experimental and control cases. It was observed also that pyruvate gave rise to  $\alpha$ -ketoglutarate when these test media were incubated aerobically. While its initial rate of formation in the maple syrup urine case was much slower than that of the control the eventual concentration attained was considerably higher. This suggested further utilization in the control tissues, and this conclusion was borne out by the progressive appearance of succinate. No succinate was formed by tissues of the maple syrup urine case.



selves, and here the normal control of their intracellular distribution may be so deranged by the high local concentrations of leucine, isoleucine and valine, that tissue function becomes seriously impaired. Whatever might be the ultimate connexion between the biochemical lesion and the clinical symptoms, the only course of treatment suggested by the present biochemical studies is the dietary restriction of the branched-chain amino acids.

Work is proceeding in an attempt to identify the substances giving rise to the maple syrup odour. It may be found that such substances are members of a wider group of compounds possessing this characteristic odour, and being derived by similar processes from intermediary acids. Smith and Strang (1958) reported a case in which the urine had a penetrating odour resembling burnt sugar, and found this property to be associated with  $\alpha$ -hydroxy-butyric acid. They suggested that the odour was due to an oxidation or polymerization product of the hydroxy acid. Similar products could also be derived from the hydroxy acids occurring in maple syrup urine.

### Summary

A case of maple syrup urine disease, with survival to 15 months, is presented. The urine contained high concentrations of leucine, isoleucine and valine, and of their respective keto acids, but other possible intermediates in the further degradation of these amino acids were not detected. The presence of the  $\alpha$ -hydroxy acid derivatives of the keto acids was confirmed.

A block in the metabolism of the three amino acids at the stage of oxidative decarboxylation is suggested by these results, but a direct demonstration

of this was prevented by the instability of the enzyme systems in storage. Some indications of a more general failure of oxidative decarboxylation were observed, and the possible significance of this and the other anomalies is discussed.

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