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^{1}H NMR Studies of Urine during Fasting: Excretion of Ketone Bodies and Acetylcarnitine

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High-resolution ^{1}H NMR spectroscopy has been applied to a study of urine from five normal human subjects during a 48-h period of fasting and for 22 h thereafter. The excretion rates of all three ketone bodies (acetoacetate, 3-D-hydroxybutyrate, and acetone), acetylcarnitine, creatinine, and sarcosine during this period were measured. Parallel increases in the excretion of the ketone bodies and acetylcarnitine were observed during fasting with little change in the output of creatinine and sarcosine. © 1986 Academic Press, Inc.

INTRODUCTION

Recent advances in nuclear magnetic resonance (NMR) spectroscopy have allowed the investigation of a wide range of increasingly complex biochemical problems. We have shown that high-resolution ^{1}H NMR spectroscopy can be used to analyze untreated biological samples such as urine and plasma. Many important intermediary metabolites can be detected by ^{1}H NMR, and quantitative information can be obtained which compares well with that from conventional analytical techniques (*1–5*).

In the present investigation we have carried out "conventional," single-pulse, ^{1}H NMR experiments on urine excreted by normal subjects during and after a 48-h fasting period and have detected and quantified ketone bodies, acetylcarnitine, creatinine, and sarcosine.

Previous studies on fasting individuals by Hoppel and Genuth (*7, 8*) reported plasma and urine concentrations of carnitine, acylcarnitines, and 3-D-hydroxybutyrate, but the analytical procedures used were lengthy and complex, and acetoacetate and acetone were not assayed (*7, 8*). The main advantage of using ^{1}H NMR for this specific application is that all three ketone bodies, acetylcarnitine, and other important excretory products can be detected rapidly and quantified simultaneously in small samples (ca. 0.5 ml) of untreated urine.

MATERIALS AND METHODS

Urine specimens were collected from healthy male subjects (aged 22–28, weight 67–82 kg) after an overnight fast, and then at regular intervals during and after a period of fasting which lasted for 48 h. During the fasting period no food was consumed,

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only water *ad libitum,* and the subjects carried out normal working schedules, i.e., were not confined to rest. All the urine excreted during the experimental period was collected. The volume and pH of each sample were recorded. pH values were all between 5.4 and 7.1. After 48 h of fasting, a light meal was consumed and urine samples were collected for a further 17 h. A second light meal was taken a further 10 to 13 h after the first (i.e., t = 58 to 61 h). Initially one subject was studied (data in Figs. 1 and 2) and subsequently a further five. However, one of the latter group suffered severe discomfort and did not continue beyond 38 h. The data from his urine are not shown but the levels of ketone bodies were much lower than expected.

^1H NMR spectra measurements. ^{1}H NMR spectra were recorded on Bruker WH400 and AM500 spectrometers (University of London facility, Queen Mary College, and MRC Biomedical NMR Centre, Mill Hill, respectively). Measurements were made on 0.45 ml of urine, with 50 μl of ^2H_2O added to provide an internal field-frequency lock for the spectrometer. This contained sodium trimethylsilyl-[$^2H_4$]-propionate (TSP) as a combined chemical shift and concentration reference (giving a final concentration of 1 m*M* TSP). The intense water signal was supressed by application of a selective secondary irradiation field at the resonance frequency of water. This was gated off during acquisition. Typically, 80 free induction decays (FIDs) were collected into 16,384 computer points. A pulse angle of 30° was used and the total delay between pulses was 5 s, to allow spin–lattice (T_1) relaxation of the protons in the sample. Assignments of ^{1}H resonances were confirmed by standard additions of candidate compounds to urine samples. Recovery studies with ketone bodies, creatinine, and acetylcarnitine were quantitative.

RESULTS AND DISCUSSION

In the initial experiment, one normal male subject was studied. Proton NMR spectra of urine collected at various times during fasting are shown in Fig. 1. Intense, characteristic resonances from acetylcarnitine and the ketone bodies (3-D-hydroxybutyrate, acetoacetate and acetone) can be identified, together with resonances from other low molecular weight metabolites which are normally present in urine (*3*). The aromatic region of the spectrum, not shown, also contains many well-resolved resonances including those for hippurate, formate, and histidine (*3*). During the course of this fasting experiment the excretion level of hippurate fell. This can be seen by the disappearance of the doublet CH_2 resonance at 4.0 ppm at 35 h (Fig. 1).

By integration of the methyl ^{1}H NMR signals for acetylcarnitine, acetone and acetoacetate, at 2.15, 2.24, and 2.34 ppm, respectively, relative to an internal standard (TSP) it was possible to quantify their urinary concentrations. Likewise, 3-D-hydroxybutyrate was quantified by integration of its doublet methyl resonance at 1.24 ppm, and creatinine and sarcosine by their *N*-methyl resonances at ca. 3.07 (shifts slightly with pH (*3*)) and 2.75 ppm, respectively. The excretion rates for these metabolites were also determined from a knowledge of the times between the collection of consecutive urine samples and their volumes. The variation of the excretion rates of selected metabolites during and after the fasting period are plotted in Figs. 2a and b. The rates of excretion of creatinine and sarcosine (Fig. 2b), two normal excretory products that can be detected in urine samples by ^{1}H NMR (*3–5*), remained relatively

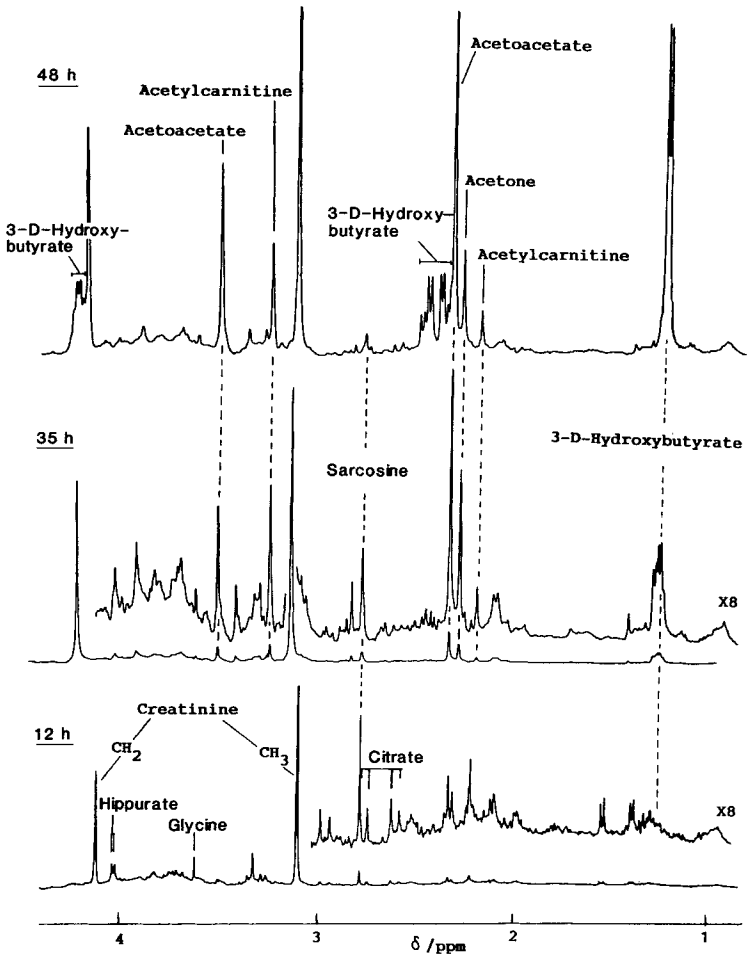

FIG. 1. 400-MHz ^{1}H NMR spectra of urine samples collected after 12, 35, and 48 h of fasting from a healthy male subject. The rise in intensity of resonances for acetoacetate, 3-D-hydroxybutyrate, acetone, and acetylcarnitine is apparent. These were calibrated with respect to a standard addition of TSP (δ = 0 ppm, resonance not shown). The data were used to calculate the excretion rates (Fig. 2) taking account of the volume of urine excreted.

constant throughout the experiment (note that the peak heights in Fig. 1 have to be scaled by the urinary volume). The average hourly excretion rate over the entire experimental period was 29.7 μmol h^{-1} for sarcosine and 560 μmol for creatinine. No individual value for sarcosine was more than $\pm$18% of this rate and only one of the individual creatinine values was greater than $\pm$15% of the mean hourly excretion rate.

There was reasonable agreement between the rate of excretion of 3-D-hydroxybutyrate during fasting obtained from ^{1}H NMR observation and those reported previously by other workers (*8*) using a conventional enzymatic assay. A concomitant increase in the excretion rates of acetylcarnitine and acetone was observed after 24 h of fasting

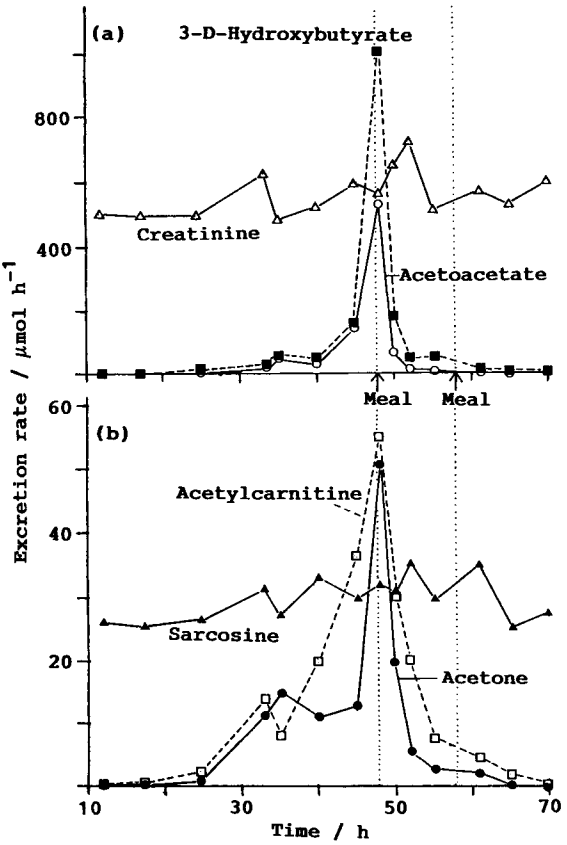

FIG. 2. The dependence of the urinary excretion rates of various metabolites (as measured by ^{1}H NMR spectroscopy) on fasting time: (a) creatinine (△), acetoacetate (○), and 3-D-hydroxybutyrate (■); (b) sarcosine (▲), acetone (●), and acetylcarnitine (□). The two vertical dotted lines indicate the times at which meals were consumed.

(Fig. 2b), although at levels an order of magnitude lower than 3-D-hydroxybutyrate and acetoacetate. The urinary acetoacetate:acetone ratio (10:1 at 48 h) was higher than has been reported previously for plasma of ketotic, fasted normal subjects (ca. 1:1 at 48 h) (*2*). It is likely that the neutral, membrane-soluble acetone molecules, produced by decarboxylation of acetoacetate, can diffuse back into the blood after glomerular filtration by the kidney and are therefore not concentrated in the urine. A similar change in this ratio has been reported for urine and plasma samples from ketotic insulin-dependent diabetic subjects studied by ^{1}H NMR (*2*).

Acetylcarnitine accounts for the major fraction of the acylcarnitines excreted during diabetic and fasting ketosis. The exact proportion depends on the obesity of the individual, but for normal thin subjects acetylcarnitine has been reported to amount to 78% of total acylcarnitines after 2 days fasting (*8*). In the present study, we were not able to assign ^{1}H NMR resonances for other acylcarnitines. Acetylcarnitine gives rise to two distinct singlet resonances, an *acetyl*-CH_3 resonance at 2.15 ppm, and also an

$N(CH_3)_3$ peak, at 3.20 ppm. The measured peak intensities for these two resonances gave the expected 1:3 ratio in all the urine samples assayed. Peak overlap of the *N*-methyl resonance with those of other compounds including other acylcarnitines did not, therefore, appear to be a problem in the samples studied. The CH_2–CH–CH_2 resonances of acetylcarnitine are complicated second-order multiplets of much lower intensities than the CH_3 resonance and were therefore not readily assignable in spectra of urine samples. No acetylcarnitine was detectable in control urine samples. However, this is not unexpected since the excretion rate is reported to be ca. 10.4 μmol^{-1} day^{-1} (*8*), which is close to the NMR detection limit. Hoppel and Genuth (*8*) have previously reported excretion levels for acetylcarnitine. Their assay was complex and involved an initial separation by ion-exchange chromatography, followed by chemical treatment with three different enzymes, and a final fluorescence assay. They reported a value of 83 μmol day^{-1} for acetylcarnitine excretion on the second day of fasting, much lower than the value of 588 μmol day^{-1} determined here in our study by ^{1}H NMR.

Within 2 h of the consumption of a light meal, there was a dramatic fall in the rate of excretion of acetoacetate, acetone, and acetylcarnitine. Seven hours after the end of the fast, only small amounts of these substances were being excreted (Fig. 2b), but significant amounts of 3-D-hydroxybutyrate were still found to be present in the urine (Fig. 2a). The rate of excretion of 3-D-hydroxybutyrate did not fall to control levels until 10 h after cessation of the fast (i.e., 58 h into the experiment), when a second light meal was taken.

These general features of the urinary excretion profiles during fasting as determined by ^{1}H NMR were confirmed with data from a further four subjects. These are shown in Fig. 3. Again the output of creatinine and sarcosine is remarkably constant and steady increases in excretion of acetylcarnitine and the ketone bodies began about 20 h into the fast. The most notable feature of Fig. 3 is the tenfold decrease in output of ketone bodies by two subjects compared to the other three examined in this paper. The physiological interpretation of this finding is currently unclear and requires further investigation. There was no obvious correlation with body build or degree of exercise taken by these two subjects. The broad picture of urinary metabolites offered by ^{1}H NMR may help to shed useful new light on physiological differences between individuals during fasting.

CONCLUSIONS

The application of ^{1}H NMR spectroscopy to the study of urine samples collected during fasting provides a rapid means of detecting all three ketone bodies and acetylcarnitine simultaneously. NMR can be quantitative and has advantages over other techniques in that it is nondestructive, requires small sample volumes, and, in certain cases, can provide confirmatory evidence of the identity of the metabolite of interest.

However, it is important to be aware of possible interferences arising from signal overlap. The acetyl ^{1}H NMR resonance for acetylcarnitine, for example, is in the same region as that for the acetyls of the drug acetaminophen and its sulfate and glucoronide conjugates 2.14–2.17 ppm (*4*).

This paper and our previous work (*2, 3*) demonstrate the potential of high-resolution ^{1}H NMR spectroscopy in studies of metabolic biochemistry. NMR has also been

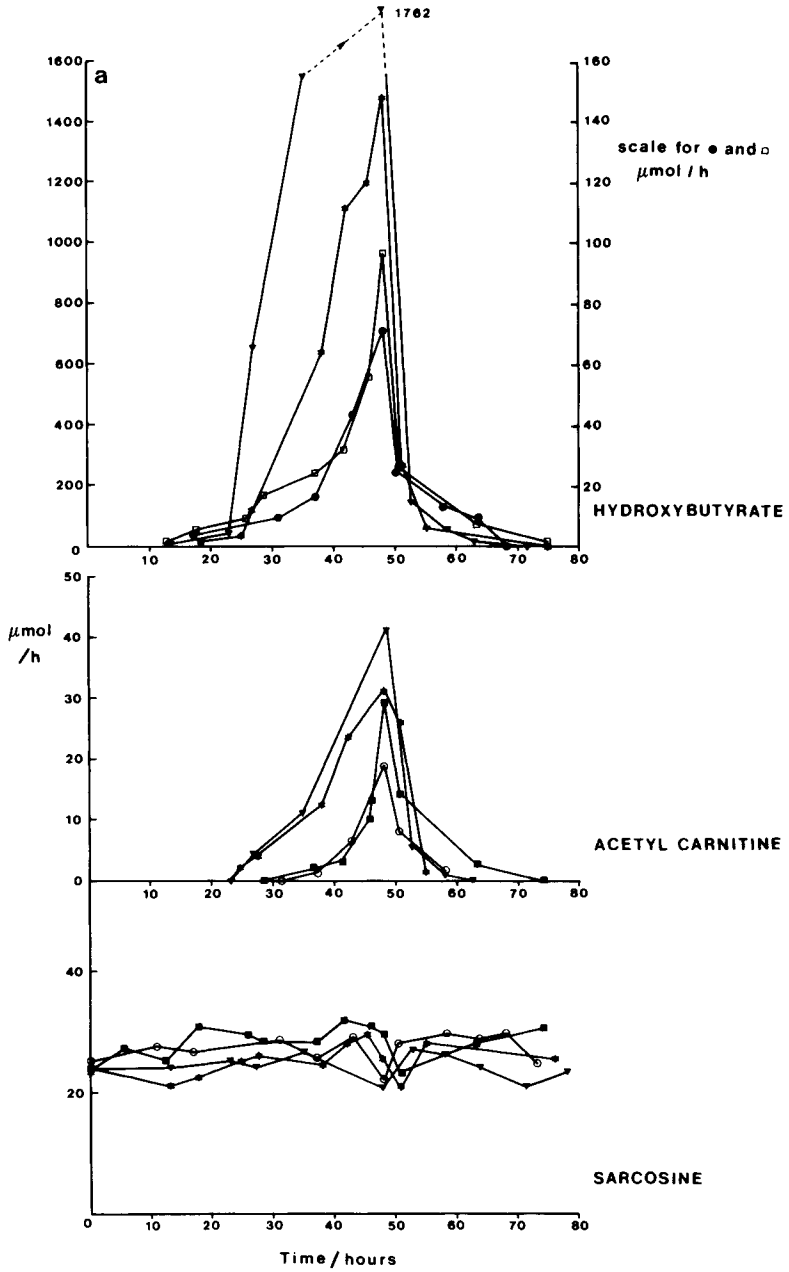

FIG. 3. Dependence of the urinary excretion rates of acetoacetate, acetone, creatinine, sarcosine, acetylcarnitine, and hydroxybutyrate on fasting time. Note that the vertical scales for the rate of excretion of ketone bodies by two subjects (● and □) are ten times less than for the other two. A light meal was consumed by all subjects after 48 h, and a second about 10 to 13 h later.

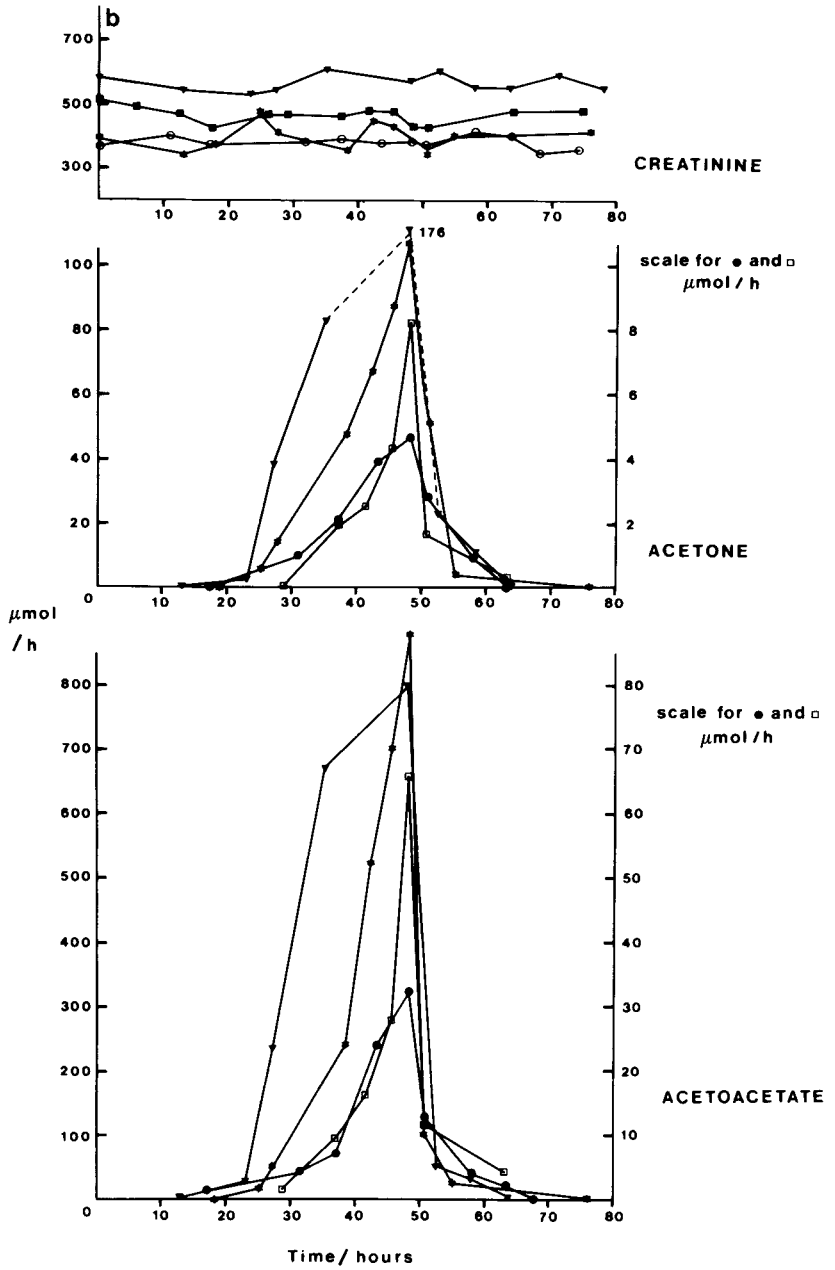

FIG. 3—*Continued.*

applied, in a similar manner, to a number of other biomedical problems including other metabolic disorders (*9, 10*), drug metabolism studies (*3, 4, 11*), and organ dysfunction (*3, 12*), and, in future, seems likely to be applicable to a wide range of clinical problems.

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