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Manifestation of Metabolic Aberration in Rat Urine Following Hypothalamic Lesions

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Abstract □ The concentrations of metabolites of low molecular weights (90 to 310 amu) present in rat urine were determined by field ionization mass spectrometry. Three groups of rats were examined; intact controls, sham-operated rats and rats with selective lesions in their hypothalamus. The latter lesions are shown to induce characteristic aberrations in the metabolic profile, demonstrable five weeks after treatment, which are distinct from those induced by a sham operation.

Keywords □ Field ionization mass spectrometry, Metabolic profile, Hypothalamic lesion, Multicomponent analysis.

Different metabolic and endocrine disturbances have been induced by electrolytic stimulation of diberent areas of hypothalamus [1,2]. It was found[1] that a chronic, diet sensitive hypercholesterolemia, without alteration in the concentration of plasma triglycerides, is promptly induced by the selective bilateral injury of the ventromedial nuclei, the fornices, and the medial portions of the lateral hypothalamic areas in the rat. The mechanism responsible for the pathogenesis of the hypercholesterolemia was found to be the retardation of the hepatic conversion of cholesterol into bile acids, accompanied by a slower removal of cholesterol from the blood stream[3~9].

The hypothalamic-lesion is expected to cause profound alterations in a complex network of metabolic interrelationships, which is expected to result in significant changes in concentrations of a large number of constituents in body fluids; however,no such extensive changes have been demonstrated to date. It seemed, therefore,of interest to measure the concentrations of low molecular weight metabolites using a methodology developed for the rapid analysis of complex biological samples[10,11]. This methodology includes column chormatography for sample preparation, field ionization mass spectrometry (FIMS) for multicomponent analysis, followed by computer-aided data analysis. The objective of the present work was to look for differences in the metabolic profiles of urines from hypothalamus-injured, sham-operated, and intact control rats. The results indicate distinctive arrays of metabolic changes associted with the hypothalamic lesion.

EXPERIMENTAL METHODS

Urine Collections

Thirty male Long-Evans rats (10 weeks old, 260-340 g weight) were randomly divided into three groups. Ten rats (group A) received two hypothalamic electrolytic lesions[9]. For the rats in group B after the surgical operation the electrodes were inserted but no electric current was passed. The remaining ten rats, which served as a control group (group C) underwent

no operation. All rats were fed 30.0ml/day of a high-cholesterol liquid diet[5] for two weeks. Plasma cholesterol level was determined at the end of the first and second week. After the second cholesterol assay, each rat was given a low-cholesterol pellet diet[5] ad lib over a period of three more weeks. Then, they were injected subcutaneously with 10.0ml of N-saline solution. Immediately after the injection, they were placed in individual metabolic cages and the urine excreted during the following 24hr period was collected. The average urine volumes of the three groups were 12.2±7.2, 12.2±8.4 and 11.9±3.2 ml, respectively. Each urine sample was stored frozen (−17°C) until it was processed for mass spectrometric analysis.

Sample Preparation and Mass Spectrometric Analysis

Rat urines were individually sampled and analyzed for their organic metabolites as described previously[11]. Briefly, the metabolites were isolated on a Chromosorb P (Supelco Inc., Bellefonte, PA. USA) column using dichloromethane as the eluting solvent, with the subsequent concentration and transfer into a miniature Chromosorb P column. The FIMS analysis was performed on a 35 cm, 60° sector magnet mass spectometer comprising a solid probe inlet, a glass lined slit type cobalt activated field ionization source, and a 4096 channel multiscaler for data accumulation. The mass range 90 to 310 amu was scanned. The integrated mass spectra were transferred to magnetic tape and then analyzed on a Burroughs 6700 computer[10].

Computer-Aided Data Analysis

Each spectrum was normalized so that the sum of all but the five largest peaks was equal to unity[11]. Duplicate molecular weight analyses were averaged, producing three groups of ten average spectra each. Due to anamalies in their cholesterol levels, the spectra representing the urine extracts of two rats were omitted from the statistical analysis sets, resulting in nine spectra for group A, ten spectra for group B, and nine spectra for group C.

The statistical analysis of the data included the Wilcoxon test[12] at each mass peak, followed by the weighted noncorrelation indices (WNI) analysis[13] comparing the individual weighted spectra to the weighted averages of group spectra.

The Wilcoxon test is often used to test the null hypothesis that two groups of data represent samples from the same population. This test was applied to each mass peak in spectra of the two groups such as A and B, A and C, and B and C to compute the significance probability, "P-value", for each mass peak. P-value is a measure of how likely it is to obtain the observed value if the two groups were indistinguishable in terms of j^{th} peak. Therefore, small P-value implies that the two groups are distinguishable in terms of the j^{th} peak. When several peaks have small P-values, we can conclude that a pattern difference exists between the two groups of spectra.

A figure of merit, "diagnostic power" (DP)[13] is derived for the significant peaks from the percentage of successful group identifications resulting from the analysis of a pair of groups where $0 \leqslant DP \leqslant 1$ as described previously[10].

WNI analysis was performed to evaluate the significance of the pattern differences. WNI were computed for individual spectra as described in detail previously[10] with the exception that the reciprocal of the P-value was chosen as a weighting factor. The resulting WNI scores of each spectrum were transformed to WNI* according to the following equation:

$$WNI_i^* = \frac{WNI_i - [\overline{WNI(A)} + \overline{WNI(B)}]/2}{[\overline{WNI(A)} - \overline{WNI(B)}]/2}$$

where, WNI_i is the WNI for the i^{th} spectrum, and $\overline{WNI(A)}$ and $\overline{WNI(B)}$ are the average WNI scores for group A and group B, respectively. This transformation normalizes the WNI of a spectrum to units of one-half the difference between the averages of the reference group WNI. It enables us to arbitrarily choose zero as a threshold of diagnostic decision for the group identification.

RESULTS AND DISCUSSION

The average plasma cholesterol levels of A,B and C groups were 99±22, 61±10 and 57±8mg/100 ml, respectively, one week postoperation, and 122±36, 61±13 and 63±17mg/100ml one week later. Seven of the ten hypothalamus-lesioned rats showed extreme elevations in cholesterol level (103 to 157 mg/100 ml) and the remaining three rats showed smaller increases (66 to 93 mg/

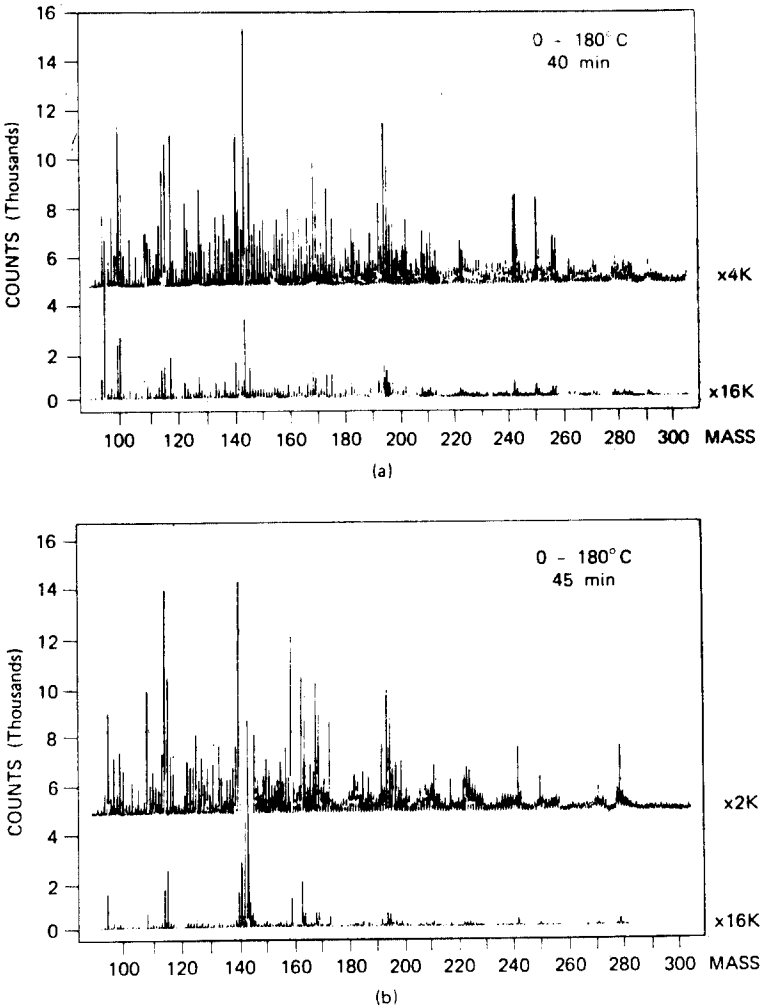

Fig. 1: Two typical raw spectra of rat urine.

100 ml). The rat with the 66 mg/100 ml was excluded from the statistical analysis of group A. No significant differences were observed in cholesterol level between sham-operated and the intact control rats. One control rat showed, however, an unaccountably high serum cholesterol level (148 mg/100 ml). This rat was therefore excluded from the statistical analysis of group C.

A total of 60 samples, duplicates of each urine sample, were analyzed in a random order. Our method gave reproducible and comparable spectral profiles with 0.50 ml of urine, as demonstrated by Figure 1. More than 200 peaks with molecular weights between 90 and 310 amu have been quantitatively measured.

An average spectrum was computed for each group, as shown in Figure 2. The $\bar{B}$ and $\bar{C}$ spectra are quite similar, whereas the $\bar{A}$ spectrum is readily distnguished, even by visual inspection, showing prominent peaks at mass numbers 250 and 298.

The Wilcoxon test was performed to compute P-value for comparisons between the two groups. The mass numbers and P-values (in percent) of peaks with the most significant differences

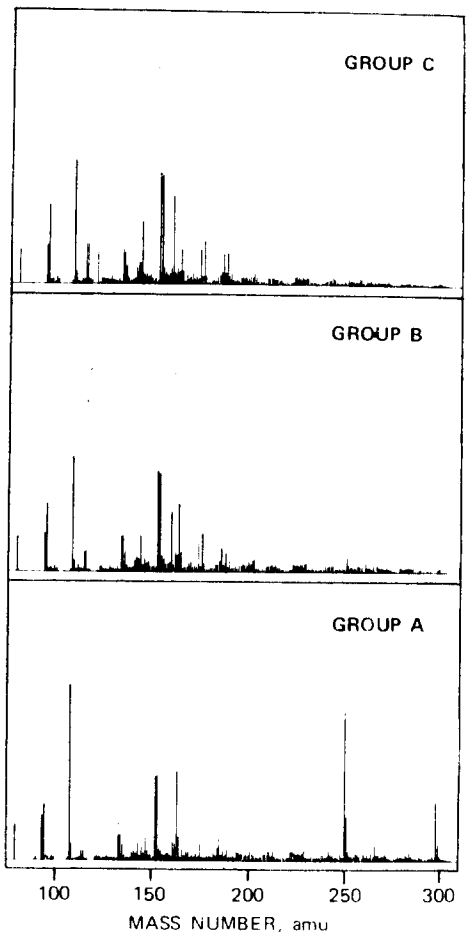

Fig. 2: Average normalized spectra.

Table I: Diagnostic power (DP) as a function of P-values for A-B comparisons.

j	m	p%	DP%
1	298	0.024	100
2	250	0.045	100
3	251	0.11	100
4	198	0.33	100
5	200	0.71	100
6	299	0.9	100
7	293	1.1	100
8	217	1.8	100
9	300	2.8	100
10	215	2.2	100
11	112	2.2	100
12	97	2.2	100
13	99	2.2	100
14	233	2.2	100
15	252	2.7	100
16	137	3.4	100
17	236	4.1	100
18	270	4.1	100
19	159	5.0	100
20	240	5.0	100
21	247	5.0	100
22	280	5.0	100
23	139	6.0	100
24	275	6.0	100
25	199	6.0	100

Table II: Diagnostic power (DP) as a function of P-values for A-C comparisons.

j	m	p%	PD%
1	250	0.035	100
2	298	0.049	100
3	251	0.067	100
4	299	0.13	100
5	139	0.13	100
6	144	0.17	100
7	143	0.23	97.5
8	125	0.31	97.5
9	160	0.31	97.5
10	300	0.31	97.5
11	296	0.41	97.5
12	112	0.41	97.5
13	186	0.41	97.5
14	252	0.41	97.5
15	233	0.54	97.5
16	128	0.54	97.5
17	159	0.71	97.5
18	236	1.2	97.5

Table III: Diagnostic power (DP) as a function of P-values for B-C comparisons.

j	m	p%	DP%
1	127	0.33	80.0
2	293	0.71	84.4
3	125	1.1	86.7
4	294	1.1	88.9
5	204	1.4	86.7
6	139	2.7	88.9
7	283	4.1	88.9
8	144	4.1	91.1
9	255	5.0	91.1
10	234	5.0	91.1

in each comparison are shown in columns 2 and 3 of Tables I, II, and III. The entire litire lists, consisting of 214 P-values, are displyed logarithmically in Figure 3.

While the B-C diflerences show relatively low

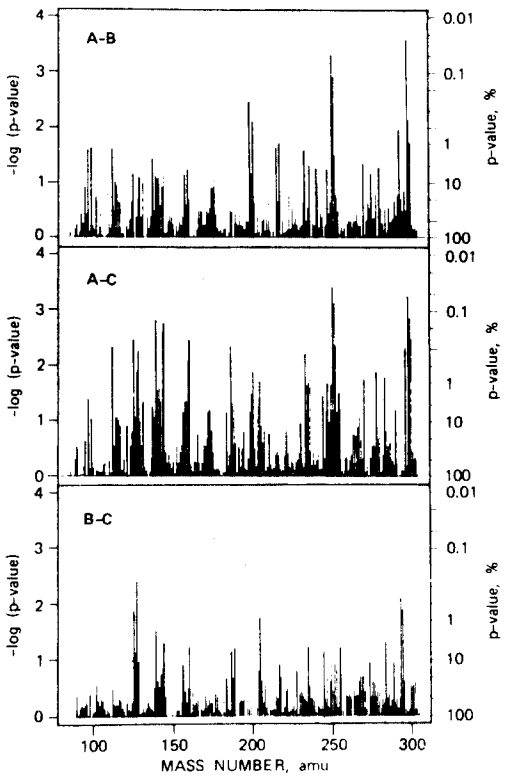

Fig. 3: P-value spectra for group comparisons.

significance, the metabolites of 125, 139, and 144 amu also appear among the significant differences in the A-C comparisons, indicating metabolic pertubations induced by the sham operation that are detectable five weeks later. The most significant amu values such as 298, 250, or 251 in column 2 of Tables I and II indicate metabolites, the concentrations of which were altered by the hypothalamic lesion.

To enhance interclass differences, the reciprocal of the P-value was used as a weighting factor in the WNI computations. It tends, however, to obscure the contribution of certain significant mass numbers, since the degree of diagnostic success will be determined, for the most part, by the spectral differences at a few mass numbers. It was decided, therefore, to

Table IV: Percent identification of numbers of a given group with established pattern of groups A,B, or C.

Test spectra	Reference pairs					
	($\bar{A}$,	$\bar{B}$)	($\bar{A}$,	$\bar{C}$)	($\bar{B}$,	$\bar{C}$)
A	100,	0	100,	0	55,	45
B	0,	100	0,	100	90,	10
C	0,	100	0,	100	11,	89
# 6	False	True	False	True	False	True
#10	False	True	False	True	False	True

compute the DP the j^{th} entries in column 4 using p_1 to p_j. It canbe seen that the two groups can be separated with higher than 95% success. B-C comparison gave less distinctive results as seen in Table III.

Each individual spectrum was then compared to each of the three reference pairs, $\bar{A}$-$\bar{B}$, $\bar{A}$-$\bar{C}$, and $\bar{B}$-$\bar{C}$ at the mass numbers corresponding to the four smallest P-values (associated with the reference pair). Table IV compiles the percentages of group identification using $T=0$ as the decision threshold for the normalized WNI. Note that, in a comparison of the spectra in group B to the reference pair $\bar{A}$, $\bar{C}$, all of the spectra were identified with $\bar{C}$, reflecting the similarity between the $\bar{B}$ and $\bar{C}$ spcetra in Figures 2 and 3. Similarly, all of the group C spectra are identified with $\bar{B}$ in the $\bar{A}$,$\bar{B}$ pair.

An interesting test of the methodology was the examination of the identity of the two rats which have been excluded from the basic classification because of their "abnormal" cholesterol levels. As can be seen in Table IV, rat #6, an intact rat with an abnormally high cholesterol level was still identified as a member of group C. In other words, this high cholesterol level was not associated with the metabolic aberrations induced by the hypothalamic lesion. Rat #10. on the other hand, which was supposed to have an electrolytically induced lesion but showed a normal cholesterol level, was identified as a member of group B (or C). In other words, its urine did not reflect any of the features associated with a hypothalamic lesion; probably in this case, by human error, no electric current was applied to the hypothalamus.

It has been known that a chronic hypercholesterolemia is caused by the previously described selective lesion of the hypothalamus. The present work confirms this observation and indicates additional distinct metabolic disorders induced by this type of hypothalamus lesion. The metabolites, which have amu values revealed by the statistical analysis as a significant molecular weight pattern, may be considered as metabolic parameters associated with the hypercholesterolemia, which are detectable five weeks after the treatment. The observed differences in urinary metabolic profiles between the sham-operated and the intact controls indicate metabolic changes induced by the surgical operation itself, distinct from those induced by the hypothalamus lesion.

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