Rf values and colour reactions of bromazepam and its metabolites in thin-layer chromatography

Substance	Solvent system I	II	III	Color reaction Dragendorff	Bratton-Marshall	Phenol reagent
Bromazepam	0.05	0.05	0.53	orange	purple[a]	—
2-Amino-5-bromobenzoylpyridine	0.74	0.60	0.85	orange	purple	—
Unknown metabolite	0.58	0.34	0.84	orange	grayish purple	blue[b] bluish gray[c]

Adsorbent: Silica Gel G (Merck). Solvent system: I, Chloroform-ether (3:1); II, Chloroform-acetone (9:1); III, Ethyl acetate. [a] Spray with the Bratton-Marshall reagent after hydrolysis by 2*N* H_2SO_4. [b] Potassium ferricyanide-ferric chloride reagent. [c] Folin-Ciocalteu reagent.

zate was adjusted to pH 8.0 and extracted with ethyl acetate. The extract was first dried over anhyd. sodium sulfate and then evaporated in vacuo. The residue was dissolved in a small amount of ethyl acetate and was subjected to thin-layer chromatography. The Table shows the Rf values and colour reactions of bromazepam and its metabolites. The residual part was chromatographed on a column of Silica Gel by successive elution with chloroform-ether (3:1). The eluate was then submitted to rechromatography on a Silica Gel column. Elution with the same solvent system and recrystallization of the eluate from chloroform yielded ca. 20 mg of an unknown metabolite. The compound was obtained as orange prisms of m.p. 204–206° (decomp.). Thin-layer chromatography with the solvent system mentioned in the Table revealed a discrete spot without tailing. Anal. calcd. for $C_{12}H_9O_2N_2Br$: C, 49.17; H, 3.09; N, 9.56. Found: C, 49.27; H, 2.95; N, 9.81. UV $\lambda_{max}^{50\%\,\text{aq. EtOH}}$ nm ($\log \varepsilon$): 238 (4.24), 280 shoulder (3.89), 410 (3.61). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 3300 (NH_2), 1630 (C=O). NMR (in DMSO-d_6) δ: 7.02 (1H, doublet, J = 2Hz, aromatic C_4–H), 7.17 (1H, doublet, J = 2Hz, aromatic C_6–H), 8.15 (4H, multiplet, pyridyl C–H, this pyridyl aromatic protone signal was not changed from the protone signal of 2-amino-5-bromobenzoylpyridine). Mass Spectrum *m/e*: 292 (M^+). From the spectral and elemental analysis data, the structure of the metabolite under investigation was assumed to be a 3-hydroxylated derivative of 2-amino-5-bromo benzoylpyridine. For the purpose of identifying the position of the hydroxy group, the synthesis of the authentic substance is now under way. It appears that the majority of the metabolite was excreted as the glucuronide conjugated form in the urine, it may be concluded that bromazepam itself, or after opening of the benzodiazepine ring, undergoes a process of hydroxylation. Details will be published in the near future.

Zusammenfassung. Es wird über Versuche zur Isolierung von Metaboliten im Harn des Kaninchens und des Hundes nach einmaliger oraler Gabe von Bromazepam in hohen Dosen berichtet. Die Ergebnisse der Elementaranalyse und des UV-, IR-, NMR- und Massenspektren deuten darauf, dass es sich um das 3-Hydroxy-Derivat des 2-Amino-5-brombenzolypyridin handelt.

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[2] Acknowledgment. The author is grateful to Dr. M. FUKUMOTO, Nippon Roche Research Center, Tokyo (Japan) for the gift of broamzepam. Thanks are also due to Prof. M. HORI and Prof. W. SADÉE for interpretation of the IR-, NMR-, and mass-spectra.

The Penetration of the Membrane of Mitochondria, obtained from Livers of Adult or Old Rats, by Different Anions

The anion permeability of mitochondrial membranes has been widely investigated. It was shown, in experiments performed by measuring mitochondrial swelling, that chloride and fumarate penetrated mitochondria poorly, but citrate, malate, phosphate, and succinate penetrated well with the aid of specific translocases. The permeability of citrate, malate and succinate also required a small amount of phosphate, in addition to which the citrate penetration required a small amount of malate[1,2].

SPENCER and LÖWENSTEIN[3] stressed the importance of citrate ion translocation from the mitochondrion into the cytoplasm to insure the normal fatty acid synthesis. ABRAHAM et al.[4] and others[5] found a decreased fatty acid synthesis in the liver cells of adult rats which developed hyperlipaemia. In the case of old animals, however, an increase instead of this decrease was observed[6]. For these reasons it seemed worth investigating the swelling of liver mitochondria obtained from normal and hyperlipaemic rats of different ages in the presence of different anions.

Methods. Experiments were made on 44 adult (4–6-month-old) and 28 old (24–26-month-old) Wistar inbred male rats kept on 15 g semisynthetic diet daily. One half of all age-groups were neurotized according to a fixed scheduled program[7]. After the completion of the pro-

[1] J. B. CHAPPELL and A. R. CROFTS, in *Regulation of Metabolic Processes in Mitochondria* (Eds. J. M. TAGER, S. PAPA, E. QUAGLIARIELLO and E. C. SLATER; Elsevier, Amsterdam 1966), vol. 7, p. 293.
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gram, the test and control animals were bled to death. The total lipoid level of the blood serum was measured by the method of ZÖLLNER and KIRSCH[8]. The livers were homogenized and mitochondria were prepared according to the method of ERNSTER and LÖW[9] at O °C by the aid of a *Zuglói refrigerated centrifuge*. The mitochondria were incubated in a solution containing 6 m*M* Tris-HCl (pH 7.4); 0.33 m*M* EDTA; 1 µg rotenone; 7.5 mg mitochondrial protein and antimycine, chloride, citrate, fumarate, L-malate, phosphate, or succinate as is indicated in the legend of figures. All of the anions were used as ammonium salts.

The swelling of the mitochondria indicated the anion penetration. This was measured by recording the absorbance at a wavelength of 520 nm in a Spektromom-360 spectrophotometer every 20 sec. The absorbance was individually normalized by regulating the diaphragm. Curves were fitted to the experimental data by the method of least squares to characterize the degree of the mito-

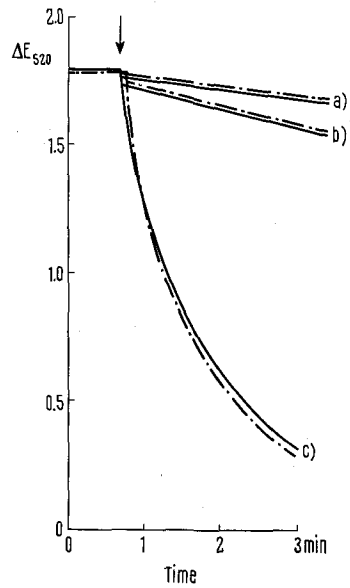

Fig. 1. The swelling of mitochondria prepared from old (dotted line) and adult (continous line) control rats in the presence of 150 m*M* chloride (a), 100 m*M* fumarate (b), or 125 m*M* phosphate (c). Anions were given at the time-point indicated by the arrow.

Table I. Effect of excitation on total serum lipoid level of rats

Rat-groups	Total serum lipoid level (mg/100 ml)	Statistical probability (p)
Adult control	270 ± 14	> 0.001
Adult neurotized	400 ± 66	
Old control	270 ± 21	> 0.001
Old neurotized	390 ± 24	

[8] N. ZÖLLNER and K. KIRSCH, Z. ges. exp. Med. *135*, 545 (1962).
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Table II. Parameters of straight lines transformed from swelling curves of mitochondria prepared from various animals in the presence of different substrates

Substrate	Rat-groups	Regression coefficient	Constant regression line	Relative error	Statistical probability of the differences
Chloride	adult	0.088	+ 1.792	0.00887	$P > 0.9$
	old	0.085	+ 1.794	0.00488	
Fumarate	adult	0.050	+ 1.797	0.00559	$0.9 > P > 0.8$
	old	0.053	+ 1.797	0.00416	
Phosphate	adult	1,485	− 0,143	0,11249	$P > 0.9$
	old	1.491	− 0.145	0.10483	
Succinate	adult neurotized	0.266	+ 0.863	0.13229	$0.9 > P 0.8$
	adult control	0.275	+ 0.855	0.13238	$0.02 > P > 0.01$
	old control	0.101	+ 1.226	0.06306	$0.9 > P > 0.8$
	old neurotized	0.105	+ 1.223	0.06746	
L-malate	adult neurotized	0.356	+ 0.522	0.18661	$0.8 > P > 0.7$
	adult control	0.351	+ 0.522	0.18350	$0.001 > P$
	old control	0.102	+ 1.226	0.06581	$0.8 > P > 0.7$
	old neurotized	0.101	+ 1.227	0.06842	
Citrate (upper descent)	adult neurotized	0.044	+ 1.519	0.00871	$t = 0.00$
	adult control	0.044	+ 0.359	0.00628	$0.2 > P > 0.1$
	old control	0.029	+ 1.431	0.01080	$0.4 > P > 0.3$
	old neurotized	0.043	+ 1.367	0.00341	
(lower descent)	adult neurotized	1.661	+ 0.361	0.12055	$0.2 > P > 0.1$
	adult control	1.933	+ 0.439	0.13890	$0.001 > P$
	old control	0.570	+ 1.082	0.04505	$0.001 > P$
	old neurotized	1.625	+ 0.565	0.17278	$0.3 > P > 0.2$[a]

[a] The probability data compare the swelling of the old hyperlipaemic rats' mitochondria with that of the adult normal rats.

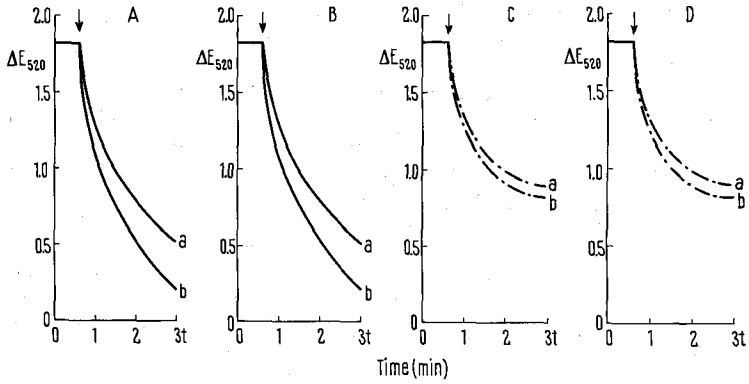

Fig. 2. The swelling of mitochondria prepared from adult control (A) and hyperlipaemic (B), or old control (C) and hyperlipaemic (D) rats in the presence of 100 m*M* succinate, 3 m*M* phosphate and 4 μg antimycine (a), or 100 m*M* malate and 3 m*M* phosphate (b). The phosphate was given at the timepoint indicated by the arrow.

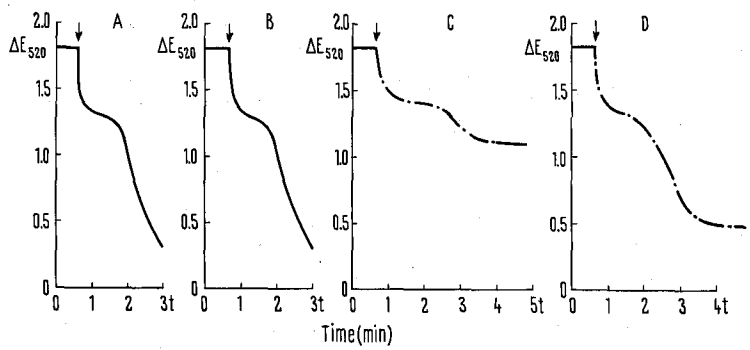

Fig. 3. The swelling of mitochondria obtained from normal and hyperlipaemic adult or old rats in the presence of 80 m*M* citrate, 2 m*M* malate and 3 m*M* phosphate. The indexes of the curves are the same as in the Figure 2. The phosphate was given at the timepoint indicated by the arrow.

chondrial swelling (Figures 1, 2 and 3). The reliability of the fittings was controlled by statistical methods. The curves were transformed into straight lines, the parameters of which are demonstrated in Table II, in order to better compare the obtained results. The probabilities were examined by the Student's *t*-test. The calculations were made by the aid of a Minsk-22 computer.

Results and discussion. The exitation caused a hyperlipaemia in the rats to a degree which was similar in both age groups (Table I). Fumarate and chloride penetrated poorly, phosphate penetrated well into mitochondria obtained from old, or adult animals without any age differences (Figure 1, Table II). In the presence of malate and succinate, the swelling of mitochondria obtained from both normal and hyperlipaemic old rats was smaller than that of mitochondria prepared from adult groups without any influence to the hyperlipaemic condition (Figure 2, Table II).

In the presence of citrate, the curves indicating the swelling of mitochondria prepared from adult rats showed a similar pattern to that found by CHAPPELL and HAARHOFF[2]. The upper descent of the swelling curves of each mitochondria indicated no significant difference. The lower descent of the swelling curves of samples obtained from normal and hyperlipaemic adult rats also showed no significant difference, but these lower descents deviated from those of samples prepared from normal and hyperlipaemic old rats: the curves indicated that the normal old rats' mitochondria hardly continued swelling, whereas the swelling of mitochondria obtained from hyperlipaemic old rats continued but to a smaller extent than that of the adult ones (Figure 3, Table II).

In regard to the difference in the swelling of mitochondria obtained from old or adult rats in the presence of di- and tricarboxylates, and to the lack of the difference in the presence of other anions, it seemed possible to assume that the activity of specific translocases is altered in the liver mitochondria of old rats.

In hyperlipaemic conditions, the fatty acid synthesis in liver cells diminished in the adult rats[4,5], but increased in that of old ones[6]. The increase of the poor penetration of citrate into the mitochondria of old rats may play a role in the latter phenomenon.

Zusammenfassung. Alter und Fettzustand von Ratten wurden variiert und die Permeabilität von Rattenleber-Mitochondrien für einige Anionen untersucht, wobei zum Teil signifikante Unterschiede festgestellt wurden.

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[11] Acknowledgment. The authors are grateful to Mrs. KATALIN DANKAI and Mr. A. SALI for the expert assistance.