Screen-printed Enzyme Electrode for the Determination of Lactose*

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An amperometric lactose sensing electrode based on thick-film technology was developed. The biosensor was based on β-galactosidase and glucose oxidase co-immobilized by cross-linking with glutaraldehyde onto the working electrodes. The enzymically generated H_2O_2 was monitored at 600 mV *versus* an Ag–AgCl reference electrode. The assay buffer was of great importance for the sensor response. Potassium citrate buffer (pH 7.0) was found to supply the best results. The buffer had to include magnesium ions, because these activate β-galactosidase. Compared with buffers without magnesium, the response increased approximately four times. The sensor showed linearity over the concentration range 2×10^{-6}–2.5×10^{-3} mol l^{-1} (correlation coefficient, $r = 0.99922$). Under optimum conditions, the sensor was stable for approximately 3 months without noticeable loss of activity. The lactose sensor was used in a batch system in order to determine the lactose content in milk and various dairy products. No interferences could be detected during the measurement of real samples and no sample pre-treatment was necessary. Sensor data for the determination of lactose were compared with those obtained by the Boehringer Mannheim test-kit method (ultraviolet method) ($r = 0.9827$, $n = 26$). The sensor method had a low relative standard deviation (8.81%, $n = 16$).

Keywords: *Lactose; thick-film technology; biosensor; immobilized enzyme; amperometric enzyme sensor*

Introduction

The characteristic carbohydrate of milk and dairy products is lactose. Lactose concentration in the milk of healthy cows is approximately 4.1–5.0%. However, milk from cows suffering from mastitis, for example, has low lactose levels. Hence lactose content is a basic indicator for evaluating milk quality and detecting abnormal milk. Besides lactose, other milk components, such as fat and protein, and hygiene parameters are important for the evaluation of milk quality.

In addition, the determination of lactose is of interest in medicine. For example, an excessive amount of lactose in blood indicates a person with gastro-intestinal malignancy. Control of the amount of lactose in foodstuffs is also important, as some persons are unable to digest the sugar fully.

Several methods are available for the determination of lactose, *e.g.*, gravimetry,[1] spectrophotometry,[2] polarimetry,[3] infrared spectroscopy,[4] titrimetry by the chloramine-T method,[5] and gas, liquid, and high-performance liquid chromatography.[6,7] Many of these methods are tedious and time-consuming, because sample preparation is complex, including extraction of lactose and filtration.

The combination of immobilized enzymes with electrochemical sensors has produced many low-cost devices. These biosensors are capable of rapid, repetitive, and sensitive assays. Some biosensors are described for the determination of lactose.[8–13] Most sensors are based on the combination of β-galactosidase (GAL) and glucose oxidase (GOD).[8,9,11–13] Another type of sensor is a uni-enzyme sensor based on galactose oxidase.[9,13] Lundbäck and Olsson[10] combined this enzyme with the enzyme peroxidase. Hamid *et al.*[13] also used mutarotase and described uni-, di-, tri-, and tetra-enzyme systems. The sensors differ in immobilization procedure. Electrochemical detection of the enzymically generated H_2O_2 is most common. With the exception of Xu and Guilbault[9] and Pilloton *et al.*,[12] all former developments are based on a flow system.

In this paper, we describe sensors that are based on immobilized enzymes, and fabricated by thick-film technology. Thick-film technology is an excellent tool for the fabrication of miniaturized electronic circuits. The technique is based on the screen printing and firing of various pastes differing in composition and electrical properties.[14] In addition, previously published papers have shown that this technology can be used successfully for the fabrication of electrochemical sensors.[15,16] It is possible to integrate working electrodes, reference, and counter electrode on one support. This is an important condition for the miniaturization of sensors.

β-Galactosidase and GOD were co-immobilized by cross-linking with glutaraldehyde on a platinum electrode. The enzymes catalysed the following reactions [eqns. (1) and (2)]:

$$\text{Lactose} + H_2O \xrightarrow{\text{GAL}} \text{D-galactose} + \text{D-glucose} \quad (1)$$

$$\text{D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \quad (2)$$

The hydrogen peroxide that is produced according to eqn. (2) was monitored electrochemically. The change of the current is proportional to the lactose concentration. The influences on the sensor response, such as buffer composition, pH, and immobilization procedures of the enzymes, are discussed. The applicability of the optimized lactose sensor for the determination of lactose in milk and dairy products without any sample pre-treatment was evaluated.

Experimental

Reagents

Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (GOD, 132 U mg^{-1} of solid using glucose as substrate) and bovine serum albumin (BSA, fraction V) were purchased from Boehringer Mannheim (Mannheim, Germany). β-Galactosidase (EC 3.2.1.23) from *Escherichia coli* (GAL, 63 U mg^{-1} of solid using lactose as substrate) was obtained from Sigma

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(Deisenhofen, Germany), glutaraldehyde (GA, 25% solution) from Merck (Darmstadt, Germany), and 3-aminopropyltriethoxysilane (ATS) from Aldrich (Steinheim, Germany). Carbohydrates were purchased from Fluka (Neu-Ulm, Germany) (lactulose and fructose), Riedel-de-Haën (Seelze, Germany) (glucose), Merck (xylose), and Sigma (maltose, sucrose, mannose, galactose, cellobiose, melibiose, raffinose, and lactose). All other chemicals were of analytical-reagent grade. Standard solutions of carbohydrates were prepared by dissolving 5 g of each sugar in 100 ml of potassium citrate buffer (pH 7.0). This solution was left to stand overnight before use, to achieve mutarotational equilibrium, and was kept in the refrigerator. The buffer concentration was always 0.1 mol l^{-1}.

The following abbreviations are used for the buffers: PPB (potassium phosphate buffer), SPB (sodium phosphate buffer), PCB (potassium citrate buffer), and SCB (sodium citrate buffer).

Thick-film Electrodes

Thick-film electrodes were fabricated in our laboratory by conventional thick-film technology. One sensor comprises four working (Pt) electrodes, a reference (Ag/Pd) electrode, and a counter-electrode (Pt). The layout and fabrication have been described elsewhere.[15]

Enzyme Immobilization

For co-immobilization of GOD and GAL, cross-linking with glutaraldehyde was used. An immobilization solution (3×10^{-6} l) containing GOD, GAL, BSA, and 15×10^{-6} l GA in 1×10^{-4} l PPB (pH 7.0) was applied to each working electrode. Different amounts of GOD, GAL, BSA, and GA were tested to obtain an optimized lactose sensor. After drying the enzyme membrane for 30 min at room temperature, the sensors were stored in assay buffer at 4 °C until used. To improve the adhesion of the enzyme layer, the electrodes were modified by silanization of the surface in a solution of ATS (10%) in distilled, de-ionized water. After washing and drying, the enzyme solution was applied to the electrode.

Assay Procedure and Apparatus

All measurements were carried out at room temperature and were achieved by immersing the sensor in 30 ml of the assay buffer. Different buffer systems and the effect of pH on the sensor response were studied to obtain optimum assay conditions.

After a steady baseline had been obtained, different amounts of the lactose standard solution were added to the buffer solution, with constant stirring. The results were used to obtain a calibration graph. The enzymically generated H_2O_2 was measured at 600 mV *versus* an Ag–AgCl reference electrode. A four-channel potentiostat (Bank, Göttingen, Germany) was used. The voltage changes were monitored and recorded against time by a four-channel chart recorder (Kipp and Zonen, Delft, The Netherlands). With real samples, sandwich additions, standard–sample–standard, were performed to eliminate possible matrix effects.

Sample Preparation

The samples could be analysed directly after homogenizing by stirring. No pre-treatment was necessary. A dilution factor of 2000 was attained by adding 15×10^{-6} l of sample to the assay buffer. As reference method, the Boehringer Mannheim test-kit lactose/D-galactose (Cat. No. 176 303, UV-method) was used. Samples were prepared by adding trichloroacetic acid for protein precipitation. After incubation, the solution was neutralized, made up to 100 ml with water, and then filtered. The clear filtrate was used for the spectrophotometric test and also for some measurements with the thick-film electrode (TFE). Raw milk samples were provided by the Forschungszentrum für Milch und Lebensmittel Weihenstephan (Freising, Germany). All other real samples were available commercially.

Results and Discussion

Optimization of Assay Conditions

To optimize the assay conditions, different buffer systems and the effect of pH on the sensor response were tested. In addition, different concentrations of magnesium and potassium ions (from $MgCl_2 \cdot 6H_2O$ and KCl) were added to the assay buffer. Unless mentioned otherwise, the studies were carried out using a carbohydrate concentration of 438.2×10^{-6} mol l^{-1}. For the soluble enzyme, it is known that magnesium and potassium ions can activate GAL from *E. coli*.[17] The activating effect of monovalent ions, especially of potassium and sodium ions, is disputed. On the other hand, activation by divalent ions such as magnesium and calcium ions is undisputed, but the extent of activation seems to depend on the system.

The effect of magnesium and potassium ions was tested in PPB and PCB as assay buffers. In both buffers, additional potassium ions had no effect, but magnesium ions increased the response to lactose approximately four times. This was only due to activation of the GAL. As shown in Fig. 1, no effect was observed when glucose was used as substrate. When lactose was used, the influence of magnesium is obvious. No influence on the sensor response was observed when 1 mmol l^{-1} calcium ions ($CaCl_2$) was added to the assay buffer. Consequently, 2 mmol l^{-1} magnesium ions were added to the assay buffer in further experiments.

The buffer used in the assay influenced the sensor response greatly. The buffers tested were potassium phosphate, sodium phosphate, Clark and Lubs buffer (CLB), sodium citrate, and potassium citrate. In all instances, the pH was 7.0, and 2 mmol l^{-1} magnesium ions were added. The best results were obtained with PCB. Using PPB or SCB, the responses were four to two times lower. Using CLB or SPB, the sensor response was greatly reduced. The presence of potassium ions appeared to be important in terms of the response, but hardly any effect was observed when potassium ions were added to SCB. The effect of pH on the response of the lactose sensor was studied from pH 5.5 to 10.0 using PPB, PCB, and CLB (Fig. 2). The optimum pH was 7.0. Hence, all measurements were carried out at pH 7.0 in PCB + 2 mmol l^{-1} Mg^{2+}.

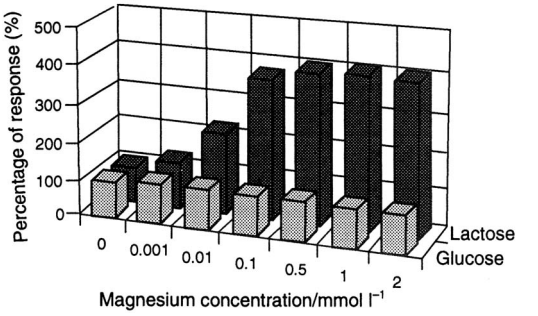

Fig. 1 Influence of magnesium ions added to the assay buffer on the sensor response. Potassium citrate buffer was used for the assay. Responses obtained without the addition of magnesium ions were set as 100%.

Optimization of Enzyme Immobilization

First, the effects of various amounts of immobilized enzymes on the sensor response were examined. For all experiments, 2.5 mg BSA and 15×10^{-6} l GA (2.5%) per 1×10^{-4} l PPB (pH 7.0) were used. Table 1 shows that the optimum ratio between GAL and GOD is 1.0:0.2 mg (63:26 U). Larger amounts of each enzyme caused a decrease in the sensor response, due to increase in thickness of the enzyme layer, which acted as a diffusion barrier. With less than 0.75–1.0 mg of GAL the result was decreased sensitivity (data not shown here) due to the low effective catalytic activity of the enzyme. As the difference in response was small over the range 0.2–0.5 mg of GOD an immobilization solution containing 0.5 mg (66 U) of GOD + 1 mg (63 U) of GAL was used for further experiments.

The optimum enzyme ratio is about 2:1 (GAL:GOD) in activity units. During enzyme immobilization, the kinetic parameters of the enzymes, K_m (Michaelis–Menten constant) and v_{max} (maximum possible chemical reaction rate of the enzyme), can change. This can be caused, for example, by conformational changes and/or partial inactivation of the enzyme.[18] Hence the kinetic parameters of the dissolved enzyme are not applicable to the immobilized enzyme. The kinetic parameters of immobilized GAL and GOD were not studied in this investigation.

The influence of the amount of BSA on the sensor response was also examined. Different amounts of BSA were added to 1×10^{-4} l immobilization solution (0.0, 2.5, 5.0 or 10.0 mg). With increasing amounts of BSA, the sensitivity towards glucose decreased. Bovine serum albumin increased the thickness of the enzyme layer, and hence the diffusion barrier effect increased with larger amounts of BSA. For lactose determinations, the sensitivity increased with the addition of BSA up to 5 mg. Using 10 mg, the sensitivity decreased compared with 5 mg of BSA. At low concentrations, BSA caused a 'gel-effect,' *i.e.*, the diffusion of large molecules was favoured over smaller ones. Fig. 3 shows the calibration graphs for lactose obtained for different amounts of BSA. The larger the amount of BSA, the wider was the linear range. With 10 mg of BSA, the sensitivity decreased. When no BSA was added, the noise was very high (signal-to-noise ratio at the detection limit, 7:1). With ≥5 mg of BSA, noise was negligible. Consequently, 5 mg of BSA were used for further experiments.

The final step in the optimization was the study of the effect of different concentrations of GA. Glutaraldehyde (15×10^{-6} l), at different concentrations (2.5, 5.0 or 10.0%), was added to 1×10^{-4} l immobilization solution. The results showed that the sensitivity for glucose and lactose decreased with increasing concentration of GA. This effect was more distinct with lactose. The more GA that was added, the lower was the sensitivity, due to the increasing thickness of the enzyme layer. A concentration of 2.5% GA was used for further experiments.

Features of the Optimum Sensor

As described earlier, the optimum immobilization solution contained 0.5 mg of GOD, 1.0 mg of GAL, 5 mg of BSA, and 15×10^{-6} l of GA (2.5%) per 1×10^{-4} l of PPB (pH 7.0). The best assay buffer, PCB (pH 7.0), was used containing 2 mmol l^{-1} magnesium ions. The calibration graph constructed for lactose was linear in the range 2×10^{-6}–2.5×10^{-3} mol l^{-1}, with a correlation coefficient of 0.99922. The sensitivity was 250 nA $mmol^{-1}$. Fig. 4 shows a typical calibration graph.

Twelve carbohydrates were tested to study the substrate specificity (Table 2). Glucose gave the highest response (set as 100%). Compared with glucose and lactose, only melibiose caused a high response; the other carbohydrates gave low

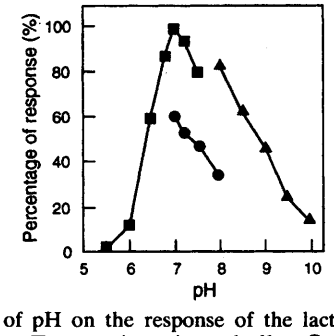

Fig. 2 Effect of pH on the response of the lactose sensor using different buffers: ■, potassium citrate buffer; ●, potassium phosphate buffer; and ▲, Clark and Lubs buffer. Lactose concentration, 2.9×10^{-4} mol l^{-1}. The maximum response was set as 100%.

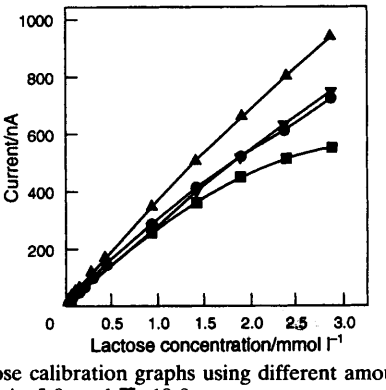

Fig. 3 Lactose calibration graphs using different amounts of BSA: ■, 0; ●, 2.5; ▲, 5.0; and ▼, 10.0 mg.

Table 1 Relationship between the enzyme content and lactose sensor response

	GAL/mg		
	1.0	1.5	2.0
	Sensor response/nA		
GOD/mg			
0.2	114.0		
0.3		100.0	
0.4			58.8
0.5	101.6		
0.75		73.3	
1.0	74.6		59.0
1.5		65.8	
2.0			62.0

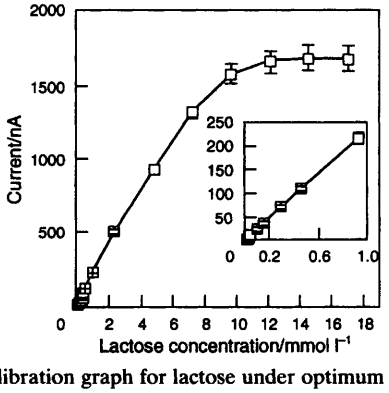

Fig. 4 Calibration graph for lactose under optimum conditions.

signals. However, as these carbohydrates are not present in milk, they would not interfere with the measurement of lactose in milk and dairy products.

Fabrication of the sensor was shown to be reproducible, the relative standard deviation (s_r) being 6.06% (n = 7).

Sensor Stability

Sensor stability depends on storage conditions. Four storage procedures were tested. The first sensor was stored under dry conditions, the second was stored in PCB (pH 7.0) with 2 mmol l^{-1} magnesium ions added, the third was kept in the same buffer without magnesium, and the fourth was stored in PPB (pH 7.0) with 2 mmol l^{-1} magnesium ions. All but the fourth sensor were used with PCB (pH 7.0) + 2 mmol l^{-1} Mg^{2+} as assay buffer. The fourth was used with PPB (pH 7.0) with 2 mmol l^{-1} Mg^{2+}. All sensors were stored at 4 °C. The results are shown in Fig. 5. Sensor 2 showed the best stability; this could be stored for nearly 3 months without noticeable loss of activity. Using PPB, considerable loss of activity was detected within a few days. This was presumably due to passivation of the electrode surface caused by components of the thick-film pastes. When the sensor was stored in the dry state, it was found to retain 25% of its initial activity after 3 weeks. After this time, the sensor activity remained constant. When stored without magnesium, the sensor lost all activity within 100 d.

Table 2 Substrate specificity of the lactose sensor. Glucose signal was set as 100%

Carbohydrate	Signal normalized to glucose
Glucose	100.0
Lactose	62.0
Lactulose	1.0
Sucrose	0.8
Melibiose	15.4
Maltose	1.3
Raffinose	0.3
Xylose	0.5
Mannose	3.5
Fructose	Negligible
Galactose	1.5
Cellobiose	1.0

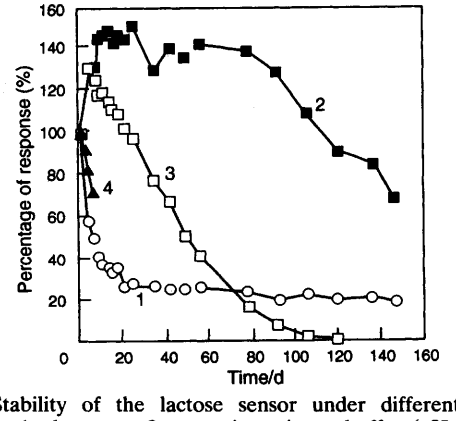

Fig. 5 Stability of the lactose sensor under different storage conditions: 1, dry state; 2, potassium citrate buffer (pH 7.0) + 2 mmol l^{-1} magnesium ions; 3, potassium citrate buffer (pH 7.0); and 4, potassium phosphate buffer (pH 7.0) + 2 mmol l^{-1} magnesium ions. Potassium citrate buffer (pH 7.0) + 2 mmol l^{-1} magnesium ions was used as assay buffer for all but the fourth sensor; this was tested in its storage buffer. Each sensor was stored at 4 °C when not in use.

With reference to the results for the immobilization of different amounts of enzymes, it is assumed that a surplus of enzymes is present in the chosen sensor and that the sensor response is mainly controlled by diffusion and not by enzyme kinetics. During storage, the enzymes lose activity, the process becomes kinetically controlled, and the response becomes smaller. By choosing the optimum storage conditions, it is possible to retain the enzyme's activity over a certain period of time, and to maintain diffusional control; the response is then stable over this period.

Analysis of Real Samples

Milk is a very complex matrix, because many interfering substances can be present. However, in fact, samples showed negligible interference during electrochemical detection. To ensure that no sample pre-treatment was necessary, the lactose content was measured in four samples, with and without sample pre-treatment. The samples were pre-treated as recommended by the reference method. The results show that no sample pre-treatment was necessary for measurements using the sensor. The results of the analysis of real samples are summarized in Table 3. The results correlate well with those obtained by the reference method (r = 0.9827, n = 26). When the proposed sensor method was applied to the determination of lactose in real samples, the reproducibility (s_r) was 8.81% (n = 16). Even after the analysis of 70 raw milk samples no loss of sensor activity was observed. As a batch system is used, no additional sample dilution was necessary. As described previously, a dilution factor of 2000 was achieved by adding the undiluted sample to the assay buffer.

Conclusion

The construction of a bi-enzyme lactose sensor based on the co-immobilization of glucose oxidase and β-galactosidase has been described. Under optimum conditions, the sensor remained stable for nearly 3 months without loss of activity. Excellent agreement was obtained between the results of analyses of milk and dairy products obtained using the sensor and those obtained by following the reference method. No sample pre-treatment was necessary. Using thick-film technology, it is possible to produce screen-printed electrodes, which can be used for the determination of lactose in milk and dairy products. The sensor is very easy to construct, and hence mass production is feasible. Because of its stability, precision, and easy handling, the sensor is convenient for industrial applications.

Table 3 Analysis of real samples based on thick-film electrodes (TFEs) and Boehringer Mannheim test-kit method

	Lactose/g per 100 g sample		
Sample	Test-kit method*	TFE*	TFE†
Full-fat milk 1	4.36	4.25	4.49
Full fat milk 2	4.34	4.13	4.73
Cream	3.15	2.95	2.69
Buttermilk	3.58	3.20	3.51
Semi-skimmed milk	4.44		4.50
Skimmed milk	4.51		4.68
Yogurt	4.21		4.11
Raw milk 1	4.59		4.74
Raw milk 2	4.33		4.16
Raw milk 3	3.17		3.06

* With sample pre-treatment.
† With no sample pre-treatment.

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References

1 *Official Methods of Analysis of the Association of Official Analytical Chemists,* ed. Horwitz, W., Association of Official Analytical Chemists, Arlington, VA, 15th edn., 1990, sect. 930.28.
2 Mistry, V. V., Hassan, H. N., and Baer, R. J., *J. Assoc. Off. Agric. Chem.*, 1989, **72**, 877.
3 Garrison, E. R., *J. Assoc. Off. Agric. Chem.*, 1939, **33**, 489.
4 Harris, W. M., *Analyst*, 1986, **111**, 37.
5 *International Dairy Federation, Standard 28A: Determination of the Lactose Content of Milk,* International Dairy Federation, Brussels, 1974.
6 Yang, M. T., Milligan, L. P., and Mathison, G. W., *J. Chromatogr.*, 1981, **209**, 316.
7 Kwak, H. S., and Jeon, I. J., *J. Food Sci.*, 1988, **53**, 975.
8 Matsumoto, K., Hamada, O., Ukeda, H., and Osajima, Y., *Agric. Biol. Chem.*, 1985, **49**, 2131.
9 Xu, Y., and Guilbault, G. G., *Enzyme Microb. Technol.*, 1990, **12**, 104.
10 Lundbäck, H., and Olsson, B., *Anal. Lett.*, 1985, **18** (B7), 871.
11 Pfeiffer, D., Ralis, E. V., Makower, A., and Scheller, F. W., *J. Chem. Tech. Biotechnol.*, 1990, **49**, 255.
12 Pilloton, R., Mascini, M., Casella, I. G., Festa, M. R., and Bottari, E., *Anal. Lett.*, 1987, **20**, 1803.
13 Hamid, J. A., Moody, G. J., and Thomas, J. D. R., *Analyst*, 1989, **114**, 1587.
14 Reichl, H., *Hybridintegration,* Hüthig Verlag, Heidelberg, 2nd edn., 1988, pp. 36–65.
15 Baumbach, P. L., *Electrochemical Sensor Construction*, UK Pat. 2073891, 1981.
16 Rüger, P., Bilitewski, U., and Schmid, R. D., *Sens. Actuators B,* 1991, **4**, 267.
17 Wallenfels, K., and Weil, R., in *The Enzymes*, ed. Boyer, P. D., Academic Press, New York, 3rd edn., 1972, vol. 7, pp. 617–663.
18 Scheller, F., and Schubert, F., *Biosensoren,* Akademie-Verlag, Berlin, 1989, pp. 52–66.

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