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Electrophoretic evidence for the impairment of complexes of the respiratory chain during iron/ascorbate induced peroxidation in isolated rat liver mitochondria

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Abstract

The impairment of the complexes of the respiratory chain was studied in isolated rat liver mitochondria under the conditions of an iron/ascorbate-mediated oxidative stress. Using blue native electrophoresis technique the NADH–ubiquinone oxidoreductase, ubiquinol–cytochrome-*c* oxidoreductase, cytochrome oxidase and ATP-synthetase were separated from mitochondrial samples at different stages of peroxidation and quantified by densitometry. In the second dimension the protein complexes were separated into their individual subunits by Tricine/SDS-electrophoresis. In relation to the time course of lipid peroxidation protein losses were moderate in the exponential phase and enhanced towards plateau phase of TBARS formation, when the intensity of staining for the native complexes became reduced by 84%, 69%, 63% and 24% for complexes I, III, V and IV, respectively, and a high molecular aggregation band as a putative marker of oxidative stress was formed. The decline of overall staining by 23%, a decrease in trichloroacetic acid precipitable protein and the formation of acid soluble primary amines suggest the occurrence of fragmentation or degradation processes. Apparently, the impairment of the respiratory chain complexes during peroxidation was not reflected in altered electrophoretic mobilities or specific losses of protein subunits of these innermitochondrial membrane components.

Keywords: Respiratory chain complex; Electrophoresis; Peroxidation; Mitochondrion; (Rat liver)

1. Introduction

The electron transport chain of mitochondria is well known for its intrinsic production of free oxygen radicals as toxic side products during respiration [1–3]. Since reactive oxygen species are likely generated in the mitochondrial inner membrane, high concentrations and profound effects of these radicals should be expected within this space. Previous studies have shown functional damages of mitochondria during oxidative stress, which are ultimately caused by a decline in the enzyme activities of the respiratory chain complexes and the ATPase [4–6].

We demonstrated a close correlation between the decreases of mitochondrial respiration and ubiquinol–cytochrome-*c*-oxidoreductase activity in the initial phase of peroxidation [5]; the other complexes behaved less sensitive against the attack of free radicals in this experimental iron/ascorbate system.

Since the mechanisms for the inactivation of the complexes of the respiratory chain are still unknown, it appeared worthwhile to study possible structural changes of the membrane proteins induced by peroxidation.

To determine the relative mitochondrial content of the complexes of respiration and oxidative phosphorylation and the subunit pattern of these protein complexes during peroxidation, we applied the recently described electrophoretic technique of Schaegger and Von Jagow [7,8],

Abbreviations: Complex I, NADH–ubiquinone oxidoreductase; complex II, succinate–ubiquinone oxidoreductase; complex III, ubiquinol–cytochrome-*c* oxidoreductase; complex IV, cytochrome oxidase; complex V, ATP-synthetase; TBARS, thiobarbituric acid reactive substances.

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which allows a separation of the mitochondrial complexes in their native form and a subsequent evaluation of the subunit pattern.

A decline of protein staining and the formation of primary amines in the course of peroxidation indicated a loss of the native complexes of the respiratory chain following in time the period of strong lipid peroxidation. Specific changes of the subunit composition of the complexes correlating to activity losses could not be established. A breakdown of membrane proteins during peroxidation is, however, evident, although it appears likely that the attack of oxygen free radicals against membrane lipids is an earlier event in the cascade of mitochondrial damage [9].

2. Methods

2.1. Preparation of mitochondria

Liver mitochondria were prepared from adult fasted Wistar rats (Shoe: WIST) by standard differential centrifugation procedure including two washing steps of the mitochondria, suspended in 0.25 M sucrose at pH 7.4 and kept at 2–4°C [10]. Mitochondrial protein was determined by a modified biuret method [11]. Functional integrity of the mitochondrial preparations was assessed by oxygraphic measurement of respiration with succinate as substrate in presence and absence of ADP [12] and the purity was checked by electron microscopy [13].

2.2. Iron / ascorbate mediated peroxidation

Freshly prepared functionally intact mitochondria were incubated in a medium containing 100 mM KCl and 10 mM Tris-HCl at pH 7.7. Mitochondrial protein in the medium was adjusted to 8 mg/ml. Oxidative stress was induced as previously described [6] by 100 μM $FeSO_4$ and 0.5 mM ascorbate. Controls were incubated without the addition of iron/ascorbate. During shaking in open air at 25°C aliquots of 200 μl were taken for electrophoresis procedure at timed intervals.

Peroxidation in the samples was stopped by addition of 10 μl DMSO and lowering the temperature to 3°C. Aliquots for TBARS determinations were quenched in trichloroacetic acid and assayed by the thiobarbituric acid procedure [14].

2.3. Electrophoresis procedures for first dimension

All following procedures were essentially done as described in detail by Schaegger and Von Jagow [7,8].

Briefly: aliquots of 200 μl peroxidized mitochondrial suspensions were solubilized by addition of 50 μl of 10% laurylmaltoside in the presence of 400 μl 0.75 M aminocaproic acid. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 2 mM. The sample was centrifuged for 30 min at 100 000 × g. The protein supernatant was supplemented with 25 μl of 5% Coomassie blue G 250 and applied to the native polyacrylamide gradient gel (5 → 13% T; 3% C) for first dimension. 1.5

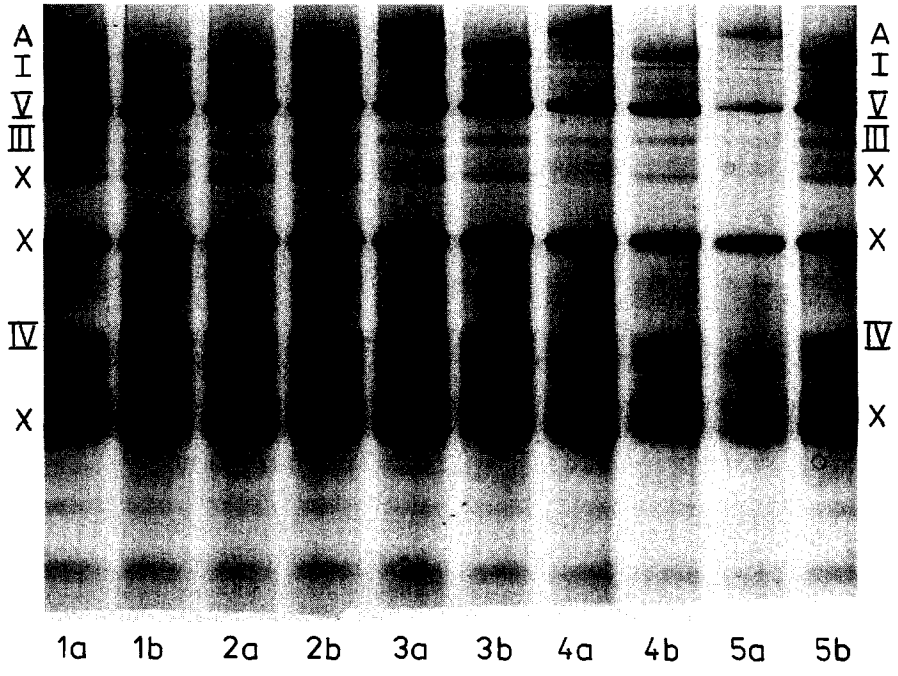

Fig. 1. Native electrophoresis patterns during iron/ascorbate induced peroxidation. 150 μl sample per lane were applied. Protein content was according to Table 1. Complex I (I); complex III (III); complex IV (IV); ATP-synthetase (V); unidentified peak (X); aggregation (A). Incubation time: 1 = 0 min; 2 = 15 min; 3 = 30 min; 4 = 60 min; 5 = 90 min. (a) 60 μM $FeSO_4$/0.5 mM ascorbate, (b) control.

mm vertical gels with a length of 15 cm were generally used. At 120 V the running time was 12 h.

After Coomassie blue G 250 staining the gels were scanned in an Ultrascan XL LASER densitometer (Pharmacia) to quantify the protein bands in each lane. To compare the mitochondrial content of the complexes I, III, IV and V during peroxidation the values were expressed as percent of the peak area of the initial content.

Protein was determined in aliquots of the supernatant after the centrifugation step by precipitation with trichloroacetic acid and addition of Lowry-reagent to the precipitate [15].

The supernatant (0.25 ml) of the trichloroacetic acid precipitation was neutralized with 1.5 ml 50 mM Hepes, pH 9.0. Primary amines were assayed by addition of 0.5 ml fluorescamine solution (0.3 mg/ml in acetone) and measured fluorimetrically using 390 nm excitation and 470 nm emission wavelength. A glycine standard curve was constructed [16].

2.4. Second dimension: Tricine-SDS-PAGE

To determine the subunit pattern of the mitochondrial complexes of oxidative phosphorylation, lanes of the native gel were separated by Tricine/SDS-electrophoresis in a second dimension. A 1 cm lane with the protein complexes was cut out of the blue native gel, dipped into a 1% mercaptoethanol solution for some seconds and placed on a glass plate at usual position of stacking gels.

After covering with the second glass plate the rest of the vertical SDS-gel was polymerized. Main component was the separating gel with 16%T, 3%C and 6 M urea. After a running time of 12 h at 100 V gels were stained by Coomassie blue G 250.

3. Results

The bands of multiprotein complexes separated in the first dimension were identified by their characteristic subunit patterns and additionally by comparison with standards [7,8]. Fig. 1 shows the separation of mitochondrial samples from different stages of peroxidation in comparison to control incubations by blue native electrophoresis. The bands corresponding to the complexes I, II, III, IV and V were identified by separating lanes of the native gel in a second dimension by Tricine/SDS-electrophoresis (Fig. 2). By means of this technique the respiratory complexes could be identified by their individual pattern of protein subunits. Thereby also the purity of each band could be checked, which is essential for the densitometric quantification later on. Prominent bands not resembling mitochondrial complexes were detected near the cytochrome-*c*-oxidase band and between complexes III and IV, but they

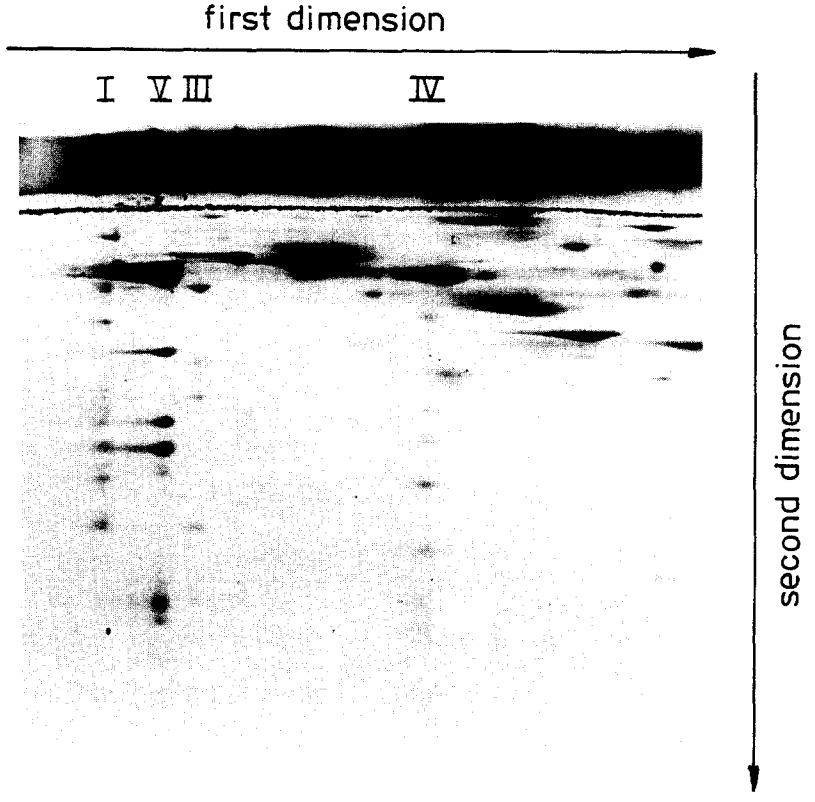

Fig. 2. Identification of native electrophoresis bands by their characteristic patterns in Tricine-SDS-PAGE (according to Schaegger and Von Jagow [6,7]). Complex I (I); complex III (III); complex IV (IV); ATP-synthetase (V).

did not interfere with the densitometric registration. Under our conditions complex II was only hardly to detect as a weak band in the native gels and in form of thin spots in the Tricine/SDS-gel. Therefore only complexes I, III, IV and V were further analyzed during peroxidation.

During incubation of mitochondria in the presence of iron/ascorbate losses in staining of all complexes could be observed in the native gels (Fig. 1). On the contrary, the intensities of the protein bands in the controls without addition of iron/ascorbate were nearly unchanged, even after 90 min of incubation.

The densitometric quantification of the protein bands in the native gels revealed that complex I exhibits the strongest decrease during peroxidation (84% after 90 min) followed by complex III (69%) and ATPase (63%). Complex IV is characterized by losses of only 24% (Fig. 3). Thus, complex IV represents the respiratory complex, which seems to be most resistant against oxidative stress. In relation to lipid peroxidation as documented by the TBARS levels, three phases of protein losses can be distinguished from each other:

(i) In an initial phase of lipid peroxidation (0–15% of the final TBARS level) only small changes in protein staining were apparent.

(ii) During the time of the exponential phase of TBARS formation (15–80% of the final TBARS level) increased, but still moderate protein losses were detected.

(iii) Towards the plateau phase of TBARS formation (80–100% of the final TBARS level) the highest rates of protein losses in all complexes were expressed (Fig. 3, Table 1).

In the course of peroxidation a new high molecular aggregation band appeared in the first dimension within the acrylamide range of 6% T (Fig. 1). This aggregation was split in a large number of fragments under the reducing conditions of the SDS-dimension (not shown), but these fragments could not be attributed to individual subunits of mitochondrial complexes. The aggregation bands seemed to represent assembles of cross-linked portions of different mitochondrial proteins, which could not be cleaved by the technique applied. The formation of this aggregation band was most expressed during the exponential phase of lipid peroxidation. As shown in Table 1, the area of the aggregation band represents, however, not more than maximally 3% of the total scanned area. Thus, the formation of this aggregation band accounts only partially for the observed decrease of the respiratory chain proteins.

The overall staining of the gels (sum of all integrated peaks in one lane) decreased by about 23% in the course of lipid peroxidation (Table 1).

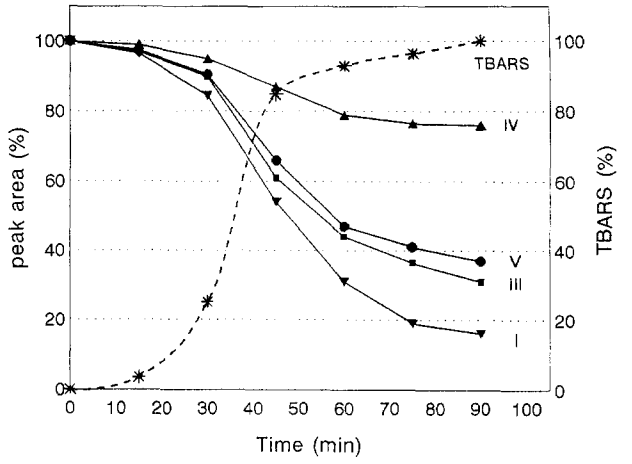

Fig. 3. Peak areas of respiratory chain complexes and TBARS formation during iron/ascorbate induced peroxidation. Complex I (I); complex III (III); complex IV (IV); ATP-synthetase (V); 100% TBARS after 90 min incubation were about 30 nmol/mg protein. 100% peak area represents the area of each complex at zero time. All values are means of five experiments with a S.D. of less than 10%. The controls did not show significant differences to the values at zero time.

Table 1
Protein content, primary amine formation and densitometric evaluation of protein staining during iron/ascorbate induced peroxidation of rat liver mitochondria

Time [a] (min)	Protein [b] (mg/ml)	Primary [c] amines (μmol/ml)	Overall [d] staining (%)	Relative staining [e] of the respiratory complexes (%)	Relative [f] staining of the aggregation band (%)
0	3.5 ± 0.20	12.2 + 0.9	100	21.4 ± 2.0	0
15	3.4 ± 0.21	13.5 ± 1.4	100 ± 1	20.7 ± 2.3	0.07 ± 0.11
30	3.1 ± 0.23	16.7 ± 0.7	95 ± 4	19.5 ± 1.7	0.43 ± 0.40
45	2.9 ± 0.25	19.2 ± 0.8	85 ± 9	16.8 ± 3.0	1.70 ± 0.61
60	2.8 ± 0.26	21.6 ± 2.0	79 ± 3	15.6 ± 2.8	2.82 ± 0.22
90	2.6 ± 0.30	25.2 ± 2.1	77 ± 2	12.8 ± 2.8	3.26 ± 0.50

[a] Incubation time. Iron/ascorbate was omitted from the controls.
[b] Represents the protein concentration in the supernatant after solubilization and centrifugation of the samples, determined by the Lowry method after trichloroacetic acid precipitation. The protein content in the controls after 90 min incubation was 3.4 ± 0.23 mg/ml.
[c] Primary amines determined by fluorescamine-reactivity in the neutralized supernatants of trichloroacetic acid precipitates from column (b). In the controls the concentration of primary amines increased to 15.0 ± 0.8 μmol/ml after 90 min incubation.
[d] Denotes to the total area under the scanned curve (% of zero time).
[e] Staining of respiratory chain complexes denotes to the sum of the peak areas of complexes I, III, IV and ATP-synthetase in relation to the overall staining area at corresponding incubation times.
[f] Peak area of aggregation band in relation to overall staining area at corresponding incubation times.
Data represent mean values ± S.D. from five experiments.

Corresponding to this, the amount of trichloroacetic acid precipitable protein in the aliquots applied to the gels decreased by 26% during the incubation (Table 1). Primary amines increased at least 2-fold in the supernatants after trichloroacetic acid precipitation, indicating the appearance of small acid soluble peptides or free amino acids (Table 1). Thus, we suppose that the decrease in staining may be mainly due to protein fragmentation or degradation. It should be mentioned that 'protein fragmentation' refers the direct chemical breakdown of proteins. The term 'protein degradation' means the hydrolysis of peptide bounds by proteinases and peptidases [26].

The portions of the sum of the identified respiratory chain proteins diminished from 21.4% in the beginning of incubation to 12.8% of total staining at the end (Table 1). It follows that these proteins of the respiratory chain account for about 50% of total protein losses during peroxidation. Since prominent smears or bands, formed as products of a partial proteolytic degradation or as a result of disassembling of subunits, were not detected in the native and Tricine/SDS-gels, respectively, these results indicate an unspecific rapid degradation of proteins and obviously a preferential attack of the mitochondrial complexes in the course of free radical reactions.

Attempts to detect specific changes in the subunit composition of the respiratory chain complexes during oxidative stress in mitochondria were not successful. The decrease of staining of the respiratory chain complexes in the first dimension was not accompanied by specific losses of single subunits in the SDS gels, as shown for complex III and ATP-synthetase in Fig. 4. Rather an uniform decline of subunit staining could be observed.

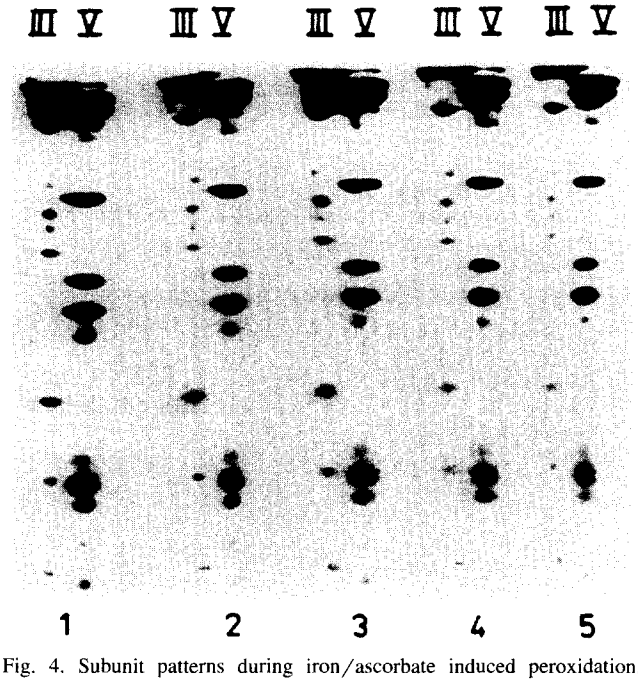

Fig. 4. Subunit patterns during iron/ascorbate induced peroxidation. Subunit patterns of complex III (III) and ATP-synthetase (V) are shown by Tricine-SDS-PAGE in second dimension. Incubation time: 1 = 15 min; 2 = 30 min; 3 = 45 min; 4 = 60 min; 5 = 75 min; representative gel from a total of six.

4. Discussion

Oxidative stress in mitochondria is answered by synergistic and consecutive reactions of different defence systems against free radical attack.

Within the lag phase of peroxidation the antioxidative defence potential of mitochondria breaks down [6,17,18]. This results in the onset of exponential increase of TBARS as an index of lipid peroxidation.

At the same time the staining of the respiratory chain complexes starts to decline (Figs. 1 and 3). The highest rate of protein loss was, however, apparent in the plateau phase of TBARS formation.

It is conceivable that the structure of membrane proteins may be 'protected' by the surrounding lipid-bilayer containing α-tocopherol. This means that a remarkable attack of free radicals to the membrane proteins may be prevented as long as they exist within their normal lipid matrix. Similar conclusions have been drawn from experiments concerning monoaminooxidase from mitochondrial outer membranes [19]. At the opposite water-soluble proteins from red blood cells were considerably degraded by proteolytic systems before any exponential increase of malondialdehyde formation was apparent [20].

The exponential phase of lipid peroxidation is characterized by the decline of all mitochondrial functions related to the regeneration of antioxidants, as glutathione and α-tocopherol [17,18], which in turn depend on the supply of hydrogen from respiratory substrates. Thus, the decline of mitochondrial respiration and the reduced activities of respiratory chain complexes are ultimately connected with the decrease in total mitochondrial antioxidative capacity. In a previous study it was shown that the inactivation of complex III during the initial phase of peroxidation is strongly correlated to the decrease of active mitochondrial respiration [5]. The results presented here point out that alterations of the mitochondrial complexes by modifications, which likely are responsible for the different reductions of enzymatic activities, are not reflected by changed electrophoretic mobilities in denaturing electrophoresis or in specific losses of protein subunits of these complexes (Fig. 4). The high susceptibility of the bc_1-complex activity in comparison to the other respiratory chain complexes is not detectable by structural alterations seen in native and Tricine-electrophoresis, as well. Complex I, which declined in its activity less than complex III during the induction phase of peroxidation [5], was, however, more sensitive to structural breakdown (Fig. 3) and in agreement with data of the literature [4,21] cytochrome-*c* oxidase was very resistant against radical attack also in our experimental system.

Thus, the losses of activities have to be explained by

specific impairment of the redox-active centers in the respiratory chain proteins, presumably by oxidative modification of amino acids [22] or alternatively by changes in the phospholipid protein interactions as shown to be essential for cardiolipin and complex III [23].

The general decline of protein staining of respiratory chain complexes was shown to occur at the late phases of peroxidative attack to mitochondria rather than in the induction phase, when the decline of activities occur.

The formation of a protein band comprising aggregated proteins during the exponential phase of lipid peroxidation (Fig. 1, Table 1) may be considered as a marker of the peroxidation process. This aggregation may be a result of either a cross-linking from denatured proteins or may be also caused by reactions of oxidatively damaged phospholipids, aldehydic lipid peroxidation products and proteins, but the amount remained relatively small under our incubation conditions.

Fragmentation or degradation of inactivated denatured and phospholipid depleted proteins or rapid disassembling of mitochondrial complexes consecutively followed by proteolysis could provide an explanation for the overall decline in protein staining in the course of an oxidative stress [24,25]. Examples for a rapid degradation of unassembled subunits of mitochondrial complexes are known from yeast and other tissues [27,28]. The decreasing level of trichloroacetic acid precipitable protein and the increase of the primary amines in the present experiments mean that the observed process does obviously result in the formation of free amino acids or small peptides. An involvement of a mitochondrial proteolytic system for the degradation of oxidized proteins, which itself is relatively resistant against oxidation, as described by Davies et al. [29], is suggested, but has to be evaluated in further experiments. Although the contribution of lysosomes as part of the proteolytic system in general can not be excluded, its proportion should be small as judged from the electronmicroscopic evaluation of the resulting preparation [13].

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