feature is demonstrated by Figure 2, which shows thermal reaction in the skin collagen of an eight month old rat. The curve with solid circle shows the loss of birefringence in a specimen mounted under a glass disc (isotonic), while the curve with open circles shows the spring mounting (fairly isometric). It is evident from the figure that keeping the specimen in isometric conditions elevates the denaturing temperature registered. This is in agreement with the investigations of FRENKELJ et al.[10] on tail tendon fibers from 6-month-old rats. They found that the denaturing temperature could be elevated somewhat if constant loads on the parallel-fibred collagen structure in hydrothermal experiments was increased. At the same time they found that the magnitude of shortening was diminished. This can be explained by the fact that denaturing means transition from an orderly state to one, in which the molecules are randomly coiled. This transition is to a certain limit prevented by imposing a more strict orientation through tension. From this it must be evident that it is not safe to draw conclusions from results of tests, where the specimen has been subjected to unknown loads during isometric testing.

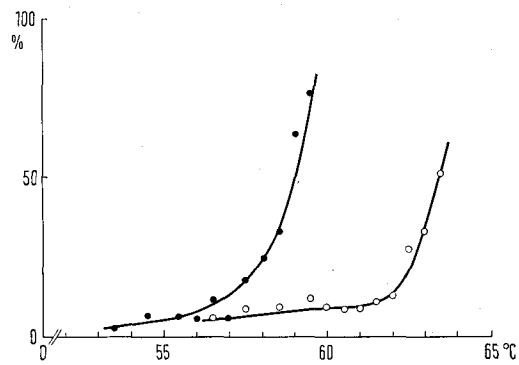

Fig. 2. Percent of initial birefringence lost with increasing temperature. Mean value of experiments on skin from an 8-month-old rat, isotonic (solid circles), and isometric (open circles) test, performed in Ringer's solution buffered to pH 7.4.

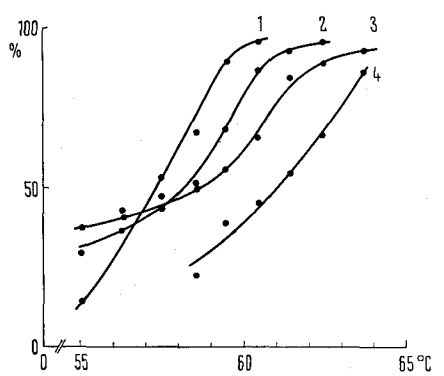

Fig. 3. Percent of initial birefringence lost with increasing temperature. Mean values of isotonic tests on human skin specimens: 1. fetus 6 months: 2. 2 months; 3. 6 years and 4. 55 years.

Discussion on the applicability of the method. To evaluate the thermal reactivity characteristics of parallel-fibred tissue, especially if the amount of it per unit specimen length is easily estimated or standardized, the isometric or isotonic method at a standard temperature is the method of choice. However, with complicated patterns of fiber arrangement or when fibers of various degrees of maturity are present, the method presented here offers advantages.

Figure 3 shows evaluation of the thermal reactivity of human skin of various ages. It seems that the thermal denaturation occurs more abruptly in the older individual than in the young one, i.e. the collagenous elements in the young one are of somewhat varying denaturing temperatures, some of which are considerably lower than those of the older skin while some of them do not differ very much. This is the same phenomenon as can be demonstrated chemically with the varying extractability of collagen (Cf. e.g. KLEIN et al.[11]).

In a healing wound, where collagens of considerably different degrees of maturation are present, the wound reaction area itself, adjacent skin and collagen distant to the site of trauma can be assessed separately (HOLM-PEDERSEN and VIIDIK[12]). It has been claimed (DOUGLAS[13]) that wound collagen has physical properties other than ordinary dermal collagen on the ground that wound collagen fails to show birefringence although it is indistinguishable from ordinary collagen by histological staining. With the methodology presented in this paper, it is shown that this 'phenomenon' is an artifact caused by the combination of histotechnique which for paraffin embedding includes heating and low denaturing temperature of young collagen. This collagen, in freezing microtome sections, exhibits the same type of birefringence as ordinary skin collagen but loses it at a considerably lower temperature than the collagen of adjacent skin, which is more mature.

Zusammenfassung. Die Messung des Denaturationsdunktes als Parameter für die thermale Reaktion des Kollagens in geometrisch komplexen Geweben oder von Kollagenen verschiedener Reifegrade wird beschrieben und Beispiele im Bereich von Altersunterschieden und Wundheilung werden gegeben.

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[14] The technical assistance of Miss ULLA-BRITT LINDGREN in the development of this method is gratefully acknowledged.

Electrophoretic Analysis and Molecular Weight Estimation of Proteins from Guinea-Pig Brain Subcellular Fractions

The isolation and characterization of the chemical components of the synaptic membranes is a prerequisite toward a complete understanding of the complex events that take place at the synaptic level. Techniques for isolation of brain subcellular particles such as 'synatosomes' have been available in the last decade[1,2] and many comparative studies have been carried out on the different fractions obtained by gradient centrifugation.

Yet very little is known about the protein components of the synaptic membranes. BOSMANN et al.[3] have recently described the extraction with sodium dodecylsulfate (SDS) of rat brain subcellular particles and their electrophoretic behaviour. In their paper the total number of protein and glycoprotein bands is reported for each fraction, but unfortunately no comparisons are made among the fractions so that, since cross-contamination is often present, it is impossible to know which bands have to be considered as characteristic components and which as contaminants.

The present communication describes a comparative electrophoretic analysis of SDS solubilized guinea-pig brain subcellular particles carried out with the aim of identifying typical components of the different fractions.

The molecular weights of the major protein bands have been extrapolated from the gel patterns by comparing their mobilities in SDS gels with those of marker proteins of known molecular weight.

Materials and methods. Myelin, synaptosomes and mitochondria were prepared from guinea-pig whole brain following the procedure of GRAY and WHITTAKER[1]. Guinea-pigs weighing 250 g were used, which had been starved 18 h before sacrifice. In some experiments mitochondrial fractions (MT_1) were prepared according to DE ROBERTIS et al.[4] with a modification in the gradient system which in our case was 1.4–1.2–1.0–0.8 *M* sucrose. Protein was determined by the method of LOWRY et al.[5] as modified by HESS and LEWIN[6]; bovine serum albumin was used as a standard.

Solubilization and electrophoresis were carried out essentially as described by LAEMMLI[7]. 70 µg of protein for each fraction were subjected to electrophoresis at 2 mA/gel for $3^1/_2$ h in 5 × 100 mm 10% polyacrylamide gels. Following electrophoresis, the gels were stained according to WEBER and OSBORN[8], and destained by electrophoresis at 10 mA/gel in 7% acetic acid. After destaining, the protein zones were quantitated with a Chromoscan densitometer (Joyce, Loebl and Co., Ltd., England).

The molecular weight determinations were done according to SHAPIRO, VIÑUELA and MAIZEL[9] and to WEBER and OSBORN[8]; bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin and cytochrome *c* were purchased from Serva, Heidelberg; deoxyribunuclease (EC 3.1.4.5.) was obtained from Worthington.

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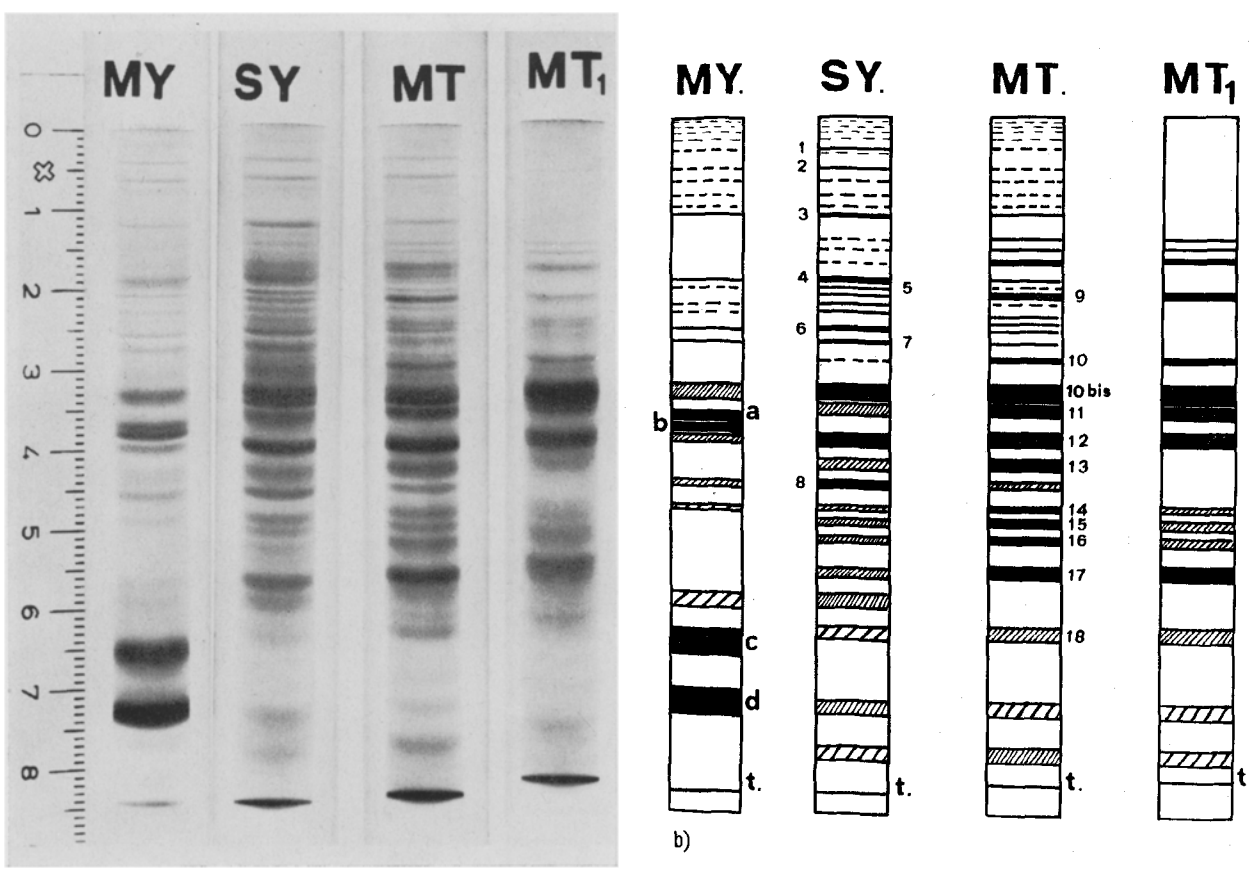

Fig. 1. a) Gel electrophoresis of SDS solubilized myelin (MY) synaptosomal (SY) and mitochondrial (MT, MT_1) fractions from guinea-pig brain; b) diagrammatic representation of the electrophoretic pattern. Migration was toward the bottom which is the anode. Cross hatched areas and dotted lines in b) indicate lightly stained and fine bands, respectively. t = tracking dye[12].

Results and discussion. Figure 1 illustrates the electrophoretic pattern of SDS solubilized myelin, synaptosomal and mitochondrial fractions from guinea-pig brain.

The myelin fraction (MY) was separated into about 30 protein bands; the synaptosomal (SY) and mitochondrial (MT) fractions had about 40 protein bands.

As far as the myelin fraction is concerned, only 4 bands (named *a, b, c* and *d*) seem to be typical components; they were not present in the other fractions and furthermore WAEHNELDT and MANDEL[10] have recently found purified rat brain myelin to be composed of 3 proteins; purified myelin from other animal species has also been reported to contain a very small number of proteins. In the densitometric tracing (Figure 2), the other protein bands showed higher intensity in SY than in MY thus suggesting a contamination of SY in MY.

Bands from 1 to 8 were common to all of the 3 fractions, but the scans shown in Figure 2 gave higher values for SY than for MY or MT. When mitochondria of higher purity (MT_1) were prepared and subjected to electrophoresis, none of the 8 bands were found, thus allowing the conclusion that bands 1–8 are typical components of SY. One could then assume that MY does not contaminate SY but SY seems to contaminate both MY and MT.

The proteins corresponding to the bands from 9 to 18 were considered as mitochondrial proteins; these bands were also present in SY fraction (which is known to contain intrasynaptosomal mitochondria) but the densitometric values were higher in MT than in SY.

The molecular weights of the major protein bands are listed in the Table. Since the molecular weight determination in 10% gels is reliable only in the range 20,000 to 80,000, in the case of SY it is only possible to say that most of its components have a molecular weight higher than

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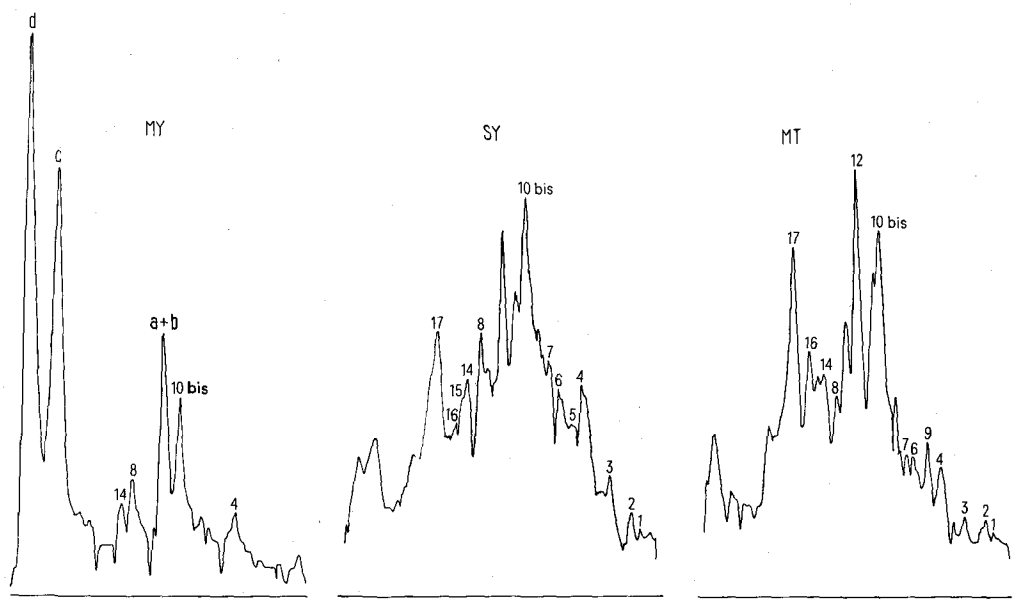

Fig. 2. Densitometric tracing of the electrophoretic patterns shown in Figure 1. MY, SY and MT indicate myelin, synaptosomal and mitochondrial fractions respectively. The bands are numbered as in Fig. 1b.

Molecular weights of myelin (MY), synaptosomal (SY) and mitochondrial (MT) proteins from guinea-pig brain determined by comparing their mobility in SDS gels with those of marker proteins with known molecular weights[8,9].

Molecular weight range	Number of bands visible in			Molecular weights of major proteins (s. Fig. 1)
	MY	SY	MT	
> 70,000	16	22	22	n.d.[a]
70,000–40,000	4	5	4	10bis = 67,000
				11 = 58,000
				a = 54,000
				b = 52,000
				12 = 45,000
40,000–20,000	5	9	8	13 = 38,000
				8 = 36,000
				17 = 26,000
				c = 21,500
< 20,000	3	3	2	n.d.[a]

[a] n.d. = not determined (in 10% gels only determinations in the range 20,000–80,000 have good reliability).

80,000. On the other hand, a better estimation was possible for most of the MT and MY major proteins, their molecular weights being within the limits of the method[11].

Riassunto. Le proteine costituenti alcune frazioni subcellulari di cervello di cavia sono state separate mediante elettroforesi su gel di poliacrilamide in un tampone contenente sodio dodecilsolfato. Nonostante le contaminazioni crociate delle varie frazioni ottenute alla ultracentrifuga é stato possibile evidenziare componenti proteiche caratteristiche delle varie frazioni. Di alcune di tali proteine é stato determinato il peso molecolare.

[11] Supported in part by a Grant from Consiglio Nazionale delle Ricerche.
[12] The excellent work of Mr. C. PERUGINI for the photograph is gratefully acknowledged.

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Visual Demonstration of Differences in Peroxidase Activity in Iron and Manganese Deficient Citrus Leaves

Peroxidase activity measurements were undertaken to differentiate between overlapping iron and manganese deficiency symptoms in various citrus leaves[1]. Iron deficiency resulted in a decrease and manganese deficiency in an increase in peroxidase activity. The peroxidase assay consisted of measuring the time required for a change of optical density in a colorimeter (from 0 to 0.4) due to pyrogallol oxidation, and was successfully applied for diagnostic purposes in crude leaf extract of citrus plants grown in greenhouses[1] and in commercial orchards[2]. Differnces in peroxidase activity between normal and iron-deficient leaves were demonstrated on the isoenzyme level as well[3]. In this communication we present results of electrophoretic separation of isoenzymes in Mn-deficient leaf extract, in comparison with normal and Fe-deficien-leaves. The opposing effects of the afore-mentioned 2 ca. tions on the peroxidase isoenzymes were demonstratedf The results of chronometric assay[4], performed on leat discs, demonstrate these differing effects on total enzyme activity.

The method used for electrophoretic separation of isoenzymes on polyacrylamide gel is described elsewhere[3]. GREGORY's[4] chronometric method involving the reaction of ascorbic acid coupled with the product of enzymic action on benzidine, was used for the chronometric reaction. Accordingly, 8–15 leaf discs, 6–8 mm diameter, cut by a cork borer, were placed in a tube in a 5 ml solution containing $0.2M$ acetate buffer of pH 5, 0.1% ascorbic acid and $0.25M$ H_2O_2. (This mixture, in a ratio of 1:1:0.5, should be prepared approximately 48 h before use. Stored in a dark glass bottle, it will remain stable for weeks.) A few drops of benzidine solution (200 mg in 25 ml of 80% ethanol) were added and the tube contents mixed. Instantaneous development of blue colour indicates complete oxidation of ascorbic acid. Time required for the appearance of the colour, or, alternatively, the intensity of colour in the different samples after a given time, can be recorded.

Figure 1 illustrates the opposing effects of the two deficiencies on peroxidase activity in isoenzyme level. Generally, the isoenzyme patterns of Sour orange (*Citrus*

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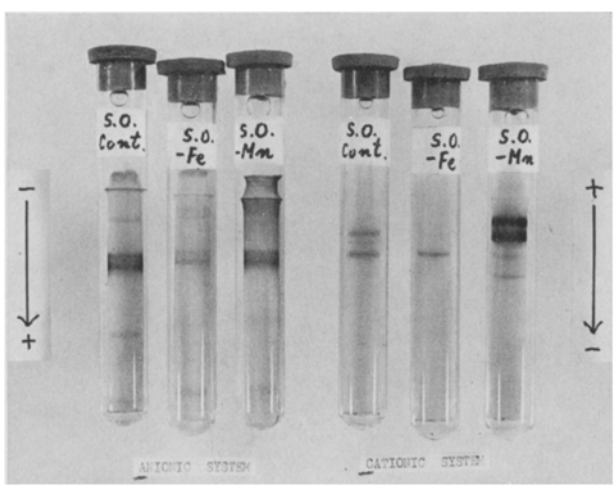

Fig. 1. Comparison of anionic and cationic peroxidase isoenzymes in full-nutrient (cont), iron and manganese deficient Sour orange leaf extract.

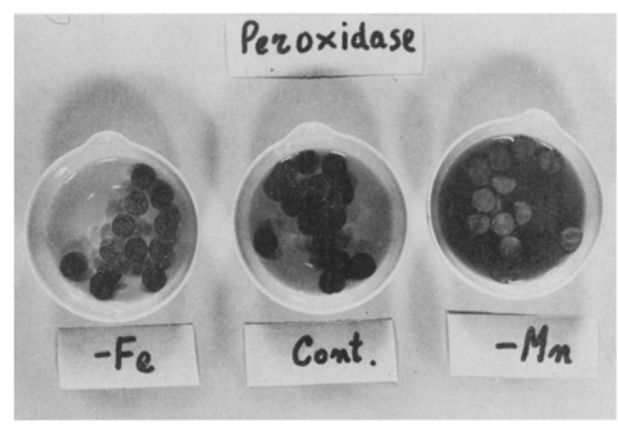

Fig. 2. Visual demonstration of differences in peroxidase activity in full nutrient (cont), iron and manganese deficient Sour orange leaf discs, by means of chronometric assay.