Histochemie 16, 68—84 (1968)

Thin Layer Chromatography and Histochemistry of Sudan Black B

A. G. W. LANSINK

Institute of Pathology of the Catholic University, Nijmegen, The Netherlands
(Head: Prof. Dr. P. H. M. SCHILLINGS)

Received June 21, 1968

Summary. Thin layer chromatography of commercial Sudan Black B on silica gel with chloroform-benzene (1:1) as the developing solvent reveals two blue main fractions with R_f values of 0.49 and 0.19, SBB-I and SBB-II respectively. Furthermore at least eighteen secondary fractions or impurities have been found. SBB-I and SBB-II were isolated and purified by preparative thin layer chromatography. Commercial Sudan Black B consists of about 20 p.c. SBB-I, 60 p.c. SBB-II and 20 p.c. secondary fractions.

From spectrophotometrical and histochemical investigations it appeared that SBB-I stains lipids more pronounced than SBB-II; moreover SBB-I is more specific for neutral lipids than SBB-II, which fraction may also stain some proteins and acid mucopolysaccharides. Contrary to SBB-II the staining with SBB-I is fairly independent of pH. Finally, the colour of SBB-II changes under the influence of light and air, while SBB-I is much more stable.

A physico-chemical study of the nature of SBB-I and SBB-II, including spectrophotometry, chromatography, infrared spectroscopy and chemical analysis revealed, that SBB-II is a basic dye, while SBB-I in spite of the structural resemblance behaves as a neutral one, dissolving therefore better in neutral lipids.

As yet the chemical composition of SBB-I and SBB-II, and the relation to the scheme of synthesis of Sudan Black B has not been solved. The unspecificity of lipid staining by Sudan Black B is due to the basicity of SBB-II, and to the instability of this dye toward light and air. Moreover the some eighteen impurities may have some influence on the staining properties. The question of solubility or adsorption processes in the case of lipid staining by Sudan dyes is at least partially answered by the proposition of a dissolving fraction SBB-I and an adsorbed fraction SBB-II. The changing absorption spectra by the corresponding solvatochromic and metachromatic effects may give information about the nature of the lipids stained.

Introduction

The composition of the common lipid stains Sudan III, Sudan IV, Oil Red 0 and Sudan Black B has been the subject of several studies (BERMES and MCDONALD, 1957; JENCKS et al., 1955; CHRISTMAN and TRUBY, 1952; KAY and WHITEHEAD, 1941; SCHOTT, 1964). In all cases a number of impurities or secondary fractions were found. Hitherto no agreement exists about the total number and the staining behaviour of fractions and impurities. KAY and WHITEHEAD (1941) and WERNER and CHRISTMAN (1952) pointed to the impurities as the most important staining fractions of Sudans III and IV. Furthermore the specificity of the lipid stains has drawn attention as the selective staining of different lipid classes (SCHOTT and SCHONER, 1965). Finally the question whether the lipid dyes, particularly Sudan Black B dissolve in the lipids or are adsorbed to the same substances has been studied by MEIER (1959) and by HOLCZINGER and BALINT (1962), who concluded to adsorption processes. Therefore solvatochromic or metachromatic effects make the interpretation of the histochemical results much more difficult.

The importance to have a stable and reproducible lipid stain has prompted the present study, in which the above mentioned problems will also be discussed.

After some preliminary experiments on some lipid stains, the investigations were continued on Sudan Black B, especially because of its great sensitiveness for phospholipids. The method of thin layer chromatography appeared to be a good and rapid one to study the Sudan dyes, and also to prepare pure fractions in rather large quantities. The staining ability of the main fractions was tested after common histological procedures. The microscopical preparations were studied by means of microspectrophotometry. In this way the physicochemical properties could be correlated with the spectrophotometrical parameters of the fractionated dyes.

Materials and Methods

All chemicals were analytical reagent grade and purchased from Merck (Darmstadt, Germany) or British Drug Houses (Poole, England). The dyes were obtained from Merck and from Gurr (London, England).

The chromatographic separations were performed on silica gel for column chromatography — below 0.08 mm and 0.05—0.20 mm — (Merck), on neutral alumina I (Woelm), on silica gel G and PF for thin layer chromatography (Merck) and on cellulose and alumina for thin layer chromatography (Camag, Muttenz, Switzerland). The analytical and preparative thin layer chromatograms were made with the Camag apparatus on glass plates of 20×10 and 20×20 cm. Preliminary and control experiments were performed with the Quickfit apparatus on plates of $15 \times 7{,}5$ cm and on microscopical object plates. Thin layer chromatography was applied following standard methods of RANDERATH (1964) and STAHL (1967), except the micro-chromatograms on object glasses which were developed and coloured according to own modifications.

Spectrophotometric maesurements were performed with the Unicam SP 800 double beam spectrophotometer and with the Zeiss PMQ II apparatus. The microspectrophotometric work was carried out with the Reichert single beam microphotometer based on the Zetopan microscope. Infrared spectra were measured with a Perkin Elmer Infracord and with the Beckman IR 4. The histological and histochemical experiments were performed on human autopsy material after fixation in neutral 10 p.c. formalin. The staining reactions were carried out according to PEARSE (1960).

Other details concerning materials and methods are reported in the next section.

Results

Thin Layer Chromatography of Sudan Black B and other Lipid Stains

Though the impurity of Sudan Black B has been recognized for some time, the number of (secondary) fractions hitherto found is limited to ten (BERMES and MCDONALD, 1957). Some preliminary thin layer experiments were aimed to yield more information about the total number of fractions of Sudan Black B, Sudan III and IV and Oil Red 0, and moreover to find the optimal combination of adsorbent and eluent for the preparative fractionation of the lipid dyes.

Comparison of the adsorbents cellulose, alumina and silica gel and also of a number of developing solvents with increasing polarity showed, that in the case of Sudan Black B an optimal separation was obtained after thin layer chromatography on silica gel with chloroform-benzene (1:1) as the eluent; otherwise the same conditions give a good separation of Sudan III and IV and of Oil Red 0 (Fig. 1). Examination of the plates above a cold light viewer, immediately after drying with air shows a large number of fractions viz. about 20 fractions of Sudan Black B, about 18 components of Sudan III, at least 10 fractions of Sudan IV, and

finally about 15 fractions in the case of Oil Red 0. From this point of view it appeared that thin layer chromatography gives a better separation of dye fractions than other methods, particularly paper and column chromatography. The most obvious feature is the presence of two main fractions in the case of Sudan Black B and Sudan IV, while Sudan III and Oil Red 0 show only one main fraction. The large sensitiveness of Sudan Black B, especially for phospholipids, the presence of the two main fractions and the question of the dissolution of this dye have led to limit the investigations on Sudan Black B, as yet.

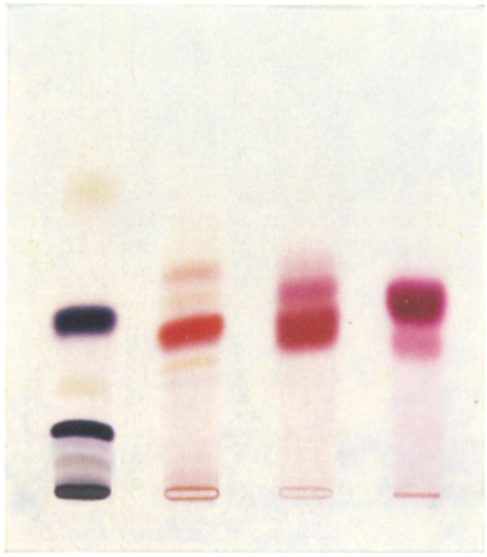

Fig. 1. Thin layer chromatogram of Sudan Black B (*SBB*), Sudan III (*SIII*), Sudan IV (*SIV*) and Oil Red 0 (*00*) on silica gel PF. Developing solvent: Chloroform-Benzene (1:1)

Preparative Thin Layer Chromatography of Sudan Black B

The necessity to have sufficient fractionated dye for both histochemical and physico-chemical experiments led naturally to the technic of preparative thin layer chromatography. Instead of silica gel G silica gel PF was used on plates of 20 × 20 cm in a layer of 1 mm. Into each plate 5 ml of a saturated filtered Sudan Black B solution in ethylalcohol was applied in a long small stroke by repetitive movement with a pipette. After drying with air and developing the plate in chloroform-benzene (1:1) the plates were dried in the air in a dark room. The silica powder with the separated fractions was scraped off with a spatula. The combined powders of some plates were treated with ethylalcohol to dissolve the dyes. After centrifugation for 20 min at about 3000 g and decantation the alcoholic dye solution was dried in a rotating vacuum evaporator at 40° C. The dyes were dissolved in acetone or ethylacetate and again dried in a vacuum exsiccator. In this manner the two main fractions could be obtained in milligram amounts a plate and in rather large purity (Fig. 2).

Moreover about 11 secondary fractions were obtained in sufficient amount for spectrophotometric examinations.

After a single preparative run the two main fractions of Sudan Black B (SBB-I and SBB-II) contain traces of impurities, as has been shown by control chromatograms. However the once fractionated dyes may be used for histochemical purposes. After a second preparative run the dyes are very pure and suitable to chemical and spectrophotometrical analysis. Control chromatograms were made in two other solvents viz. dichloroethane and ethylacetate. From the control chromatograms the conclusion was drawn at the same time, that the two main fractions exist separately in the commercial dye. Conversion of SBB-I in SBB-II or the reversed process may be ruled out.

Some preparative thin layer chromatograms were semi-quantitatively analysed by relation of the weighed amounts of the completely eluted main fractions to the known amount of dye, which has been applied to the plates. It appeared, that the commercial dye contained about 20 per cent SBB-I and 60 per cent SBB-II, the remaining 20 per cent being the secondary fractions and impurities.

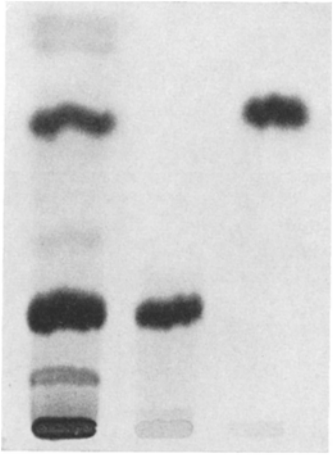

Fig. 2. Control thin layer chromatogram of the main fractions of Sudan Black B: *SBB-I* and *SBB-II*, compared with the commercial product, *SBB-T*. Adsorbent: Silica gel G. Developing solvent: Chloroform-Benzene (1:1)

Characterization of the Fractions of Sudan Black B

The fractions obtained by preparative thin layer chromatography from commercial Sudan Black B (Merck) were characterized by their R_f-values and by spectrophotometric parameters including the overall colour. The results have been summarized in Table 1.

From these data the conclusion was drawn, that the two main fractions SBB-I and SBB-II have a large resemblance of absorption spectra (Fig. 3) and probably of chemical structure. Furthermore some secondary fractions seem related to the main fractions. Otherwise a number of components are yellow, orange, brown and green in correspondence with some column chromatographic fractions of BERMES and MCDONALD (1957) and SCHOTT (1964). Returning to fractions SBB-I and -II, both the similarity and the difference of the spectra of these dyes may be read from Fig. 3. The differences are particularly situated in the regio between 400 and 500 nm. A quantitative measure to characterize the differences is taking the quotient of the extinctions at 425 and 475 nm, or at one of these wavelengths and 600 nm. However microspectrophotometry at 600 nm is difficult because of the large extinction values of tissue sections.

Table 1. *R_f-values and spectrophotometric parameters of TLC-fractions from commercial Sudan Black B*

No.	Colour	R_f	Maxima in nm	Minima in nm	E_{425}/E_{475}	Comments
1	orange	0.74	575 388	460	1.42	
2	blue	0.71	570 378	455	1.38	X
3	yellow	0.69	(575) 375	(550)	1.39	
4	orange	0.65	470		0.82	
5	green	0.55	596 398	490	1.49	X
6	blue	0.49	595 412	465 370	1.37	= SBB-I
7	yellow	0.39	(590) 348	(530)	1.23	
8	orange	0.30	(594) 425	(555) 358	1.20	
9	blue	0.27	596 400	495 375	1.41	X
10	blue	0.19	598 425	465 377	1.14	= SBB-II
11	brown	0.10	505 361	407 352	0.64	
12	blue	0.04	592 (400)	(400) 464	1.09	X
13	brown	0.00	no characteristic points		1.15	"insoluble"

R_f-values were measured after thin layer chromatography on silica gel PF 254 with chloroform-benzene (1:1) as developing solvent. The R_f-values of different runs show some variation.

All absorption spectra were measured after dissolving the dyes in 70 p.c. (v/v) ethylalcohol.

Fractions X are spectrophotometrically related to the main fractions SBB-I and SBB-II.

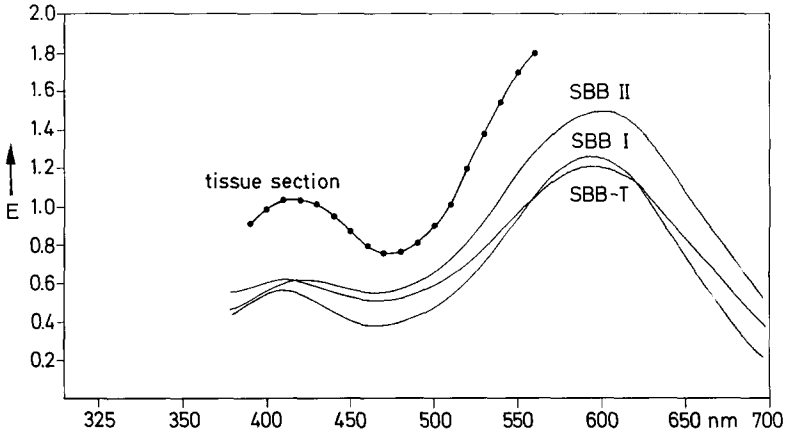

Fig. 3. Absorption spectra of fractions SBB-I and SBB-II compared with the spectra of commercial Sudan Black B (SBB-T) and of lipid droplets in a 6 mu tissue section of liver steatosis after staining with SBB-T. The spectra of SBB-T, SBB-I and SBB-II have been measured after dissolving 2.4, 2.5 and 2.9 mg dye in 100 ml 70 p.c. ethylalcohol respectively

The extinctionquotients E_{425}/E_{475} of SBB-I and -II and of SBB-T (= Sudan Black B total) in various solvents are presented in Table 2. From these data the solvatochromic effects of the dyes may be read by taking the differences of the extinctionquotients in different solvents.

Furthermore these data show the difference between the spectra of SBB-I and SBB-II and the agreement between the extinctionquotients for SBB-T, measured on a solution of the commercial product and calculated, starting from

Table 2. *Extinctionquotients E_{425}/E_{475} of SBB-I, SBB-II and SBB-T (commercial) in various solvents*

Solvent dye	Benzene	Ethyl-acetate	Acetone	70 p. c. Ethanol	Aceton/Ethanol (1:1)
SBB-I	1.48	1.39	1.46	1.36	1.44
SBB-II	1.09	1.25	1.30	1.13	1.23
SBB-T	1.17	1.28	1.34	1.18	1.26
SBB-T calc	1.19	1.28	1.34	1.19	1.28

Measurements were made by dissolving such an amount of dye, that the extinction measured in a 1 cm cuvette amounted to about 1.5 at 600 nm. The extinctionquotient for SBB-T calc (calculated) = $(E_{425}/E_{475}$ SBB-I $+ 3E_{425}/E_{475}$ SBB-II$)/4$.

the weight percents of 20:60:20 for SBB-I, SBB-II and the other fractions, the last ones being neglected.

Another extinctionquotient viz. E_{260}/E_{600} has been determined to correlate the number of benzene rings with their absorption maximum round 260 nm with the azobonds which are mainly responsible for the absorption maximum round 600 nm. The values of 0.52 and 0.57 for SBB-I and SBB-II respectively give support to the view, that the bruto structure of both dyes is about the same. Probably the differences concern only sidegroups of the dyes.

As regards the chromatographic parameters, the R_f-values also give some information about the dyes concerned. A low R_f-value (Table 1) points to a more or less ionised or at least dipolic molecule, on the contrary a high R_f-value agrees with a non-ionic hydrophobic molecule, when the separation has been performed on silica gel with a moderately polarized developing solvent. So SBB-I is less ionised or dipolic than SBB-II. The nature of the secondary fractions with yellow, red and green colour has not been investigated. Nevertheless, the chemical characterization of these substances is important in connection with the study of the synthesis scheme of Sudan Black B.

Histochemistry of the Main Fractions SBB-I and SBB-II

The evaluation of the histochemical applications of the purified main fractions SBB-I and SBB-II means a determination of the reaction with lipids in tissue sections, in relation to the same reaction of the commercial product. A quantitative evaluation of lipid staining by Sudan Black B is possible by measurement of the absorption spectrum of a lipid droplet in a tissue section after staining in a dye solution. An example of such a spectrum, measured with a Reichert microphotometer, has been given in Fig. 3. The investigation of this figure shows the correspondence of the spectra of an alcoholic SBB-I solution and of a Sudan Black B (SBB-T) stained lipid spot in a section of liver steatosis. Apparently the lipid droplet takes from the commercial dye solution, consisting of SBB-I, SBB-II and impurities only SBB-I. The preferential staining of these fat liver with SBB-I may also be read from Table 3, summarizing some microphotometric determinations of the extinctionquotients E_{425}/E_{475} of liver tissue sections after staining in SBB-I, SBB-II and SBB-T during different times. A qualitative picture of the same tissue sections is given in Figs. 4 and 5.

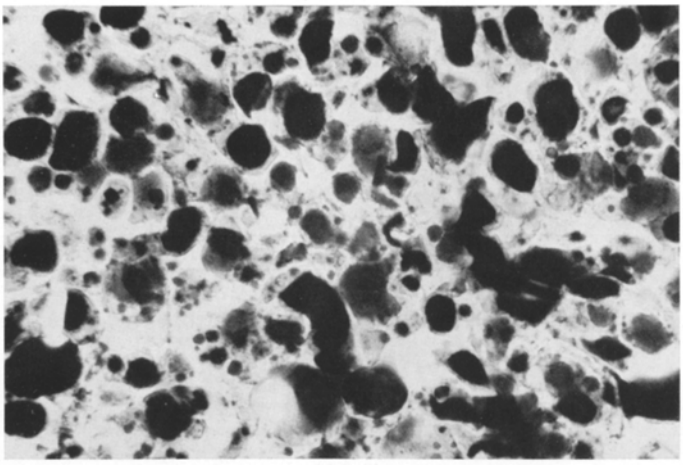

Fig. 4. Tissue section of human liver steatosis after staining in a 0.1 p.c. SBB-I solution in 70 p.c. ethylalcohol for 30 min

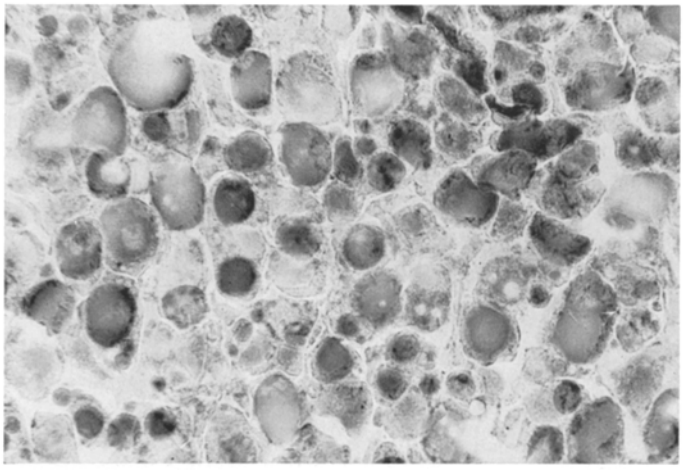

Fig. 5. Tissue section of human liver steatosis after staining in a 0.1 p.c. SBB-II solution in 70 p.c. ethylalcohol for 30 min

Table 3. *Extinctionvalues and extinctionquotients of fat liver sections after staining in SBB-I, SBB-II and SBB-T (commercial)*

Dye	Staining time	E_{425}	E_{475}	E_{600}	E_{425}/E_{475}	Standard deviation
SBB-I	30 min	1.34	0.93	1.62	1.42	0.07
SBB-I	60 min	1.36	0.98	1.66	1.38	0.06
SBB-II	30 min	0.56	0.45	1.18	1.25	0.03
SBB-II	60 min	0.67	0.55	1.25	1.22	0.07
SBB-T	30 min	0.87	0.62	1.60	1.40	0.03
SBB-T	60 min	1.14	0.84	1.70	1.37	0.04

The extinctionvalues are mean values of about 10 determinations in 2 equally treated tissue sections of 6 mu. The standard deviations have been calculated for the extinctionquotients from one set of measurements. The extinctions were measured on lipid droplets of about equal diameter.

From this experiment the following conclusions have been drawn: SBB-I stains the neutral lipids of a fat liver already after 30 min, while SBB-II has only partially dissolved or reacted with the lipids after 60 min. The quotients E_{425}/E_{475} of the stained lipid droplets correspond with the extinctionquotients of SBB-I and SBB-II in solvents (Table 2). As in these investigations the total dye concentration was equal, it appears also, that in the case of the commercial dye SBB-I dissolves or reacts with the lipids preferentially, though the reaction is not complete because of the relative small supply of SBB-I. The same experiments were furthermore carried out with some other lipid containing tissues with equal results. The extinctionquotients of some tissue sections after staining with SBB-I and SBB-II are given in Table 4.

Table 4. *Extinctionquotients E_{425}/E_{475} of tissue sections after lipid staining with SBB-I and SBB-II*

Organ	Details	E_{425}/E_{475}-SBB-I	E_{425}/E_{475}-SBB-II
Liver	Steatosis	1.40	1.23
Brain	Ischemic necrosis	1.47	1.18
Spinal cord	Fibres white matter	1.54	1.20
Spinal cord	Lipofuscine	1.60	1.24
Kidney	Nephritis	1.46	1.25
Nerves	Myelin	1.50	1.22

The extinction measurements have been carried out as mentioned with Table 3.

From the data of Tables 3 and 4 the conclusion has been drawn, that both SBB-I and SBB-II dissolve in or react with lipids of tissue sections. The microspectrophotometer makes a recognition of the dye possible if pure dyes are used. The importance of this finding will be discussed when the difference between SBB-I and SBB-II has been evaluated. As the question of the solution or the reaction of the dyes in or with the lipids has not been answered hitherto, the differences between the extinctionquotients, so far as significant shifts are concerned, give information about solvatochromic or metachromatic effects. However in both cases the shift depends not only on dye used, but also on the lipid reacting with the dye. When working with pure dye solutions the determination of extinctionquotients may give indications for the lipids in a special tissue section. The present investigation allows no definite conclusions in this respect, as parallel chemical studies of the lipid concerned have to be carried out (LANSINK, 1969).

Some Physico-Chemical Experiments on the Main Fractions SBB-I and SBB-II

The radical differences between SBB-I and SBB-II, as revealed by the above mentioned investigations and predicted by the difference of their R_f-values in chloroform-benzene (1:1), has to be linked up with the chemical structure by further physico-chemical experiments. First the evaluation of the absorption spectra of SBB-I and SBB-II as a function of pH (Fig. 6) reveals, that SBB-I is independent of pH between pH-values of about 3 and 12, whereas SBB-II shows a different absorption spectrum in acid conditions.

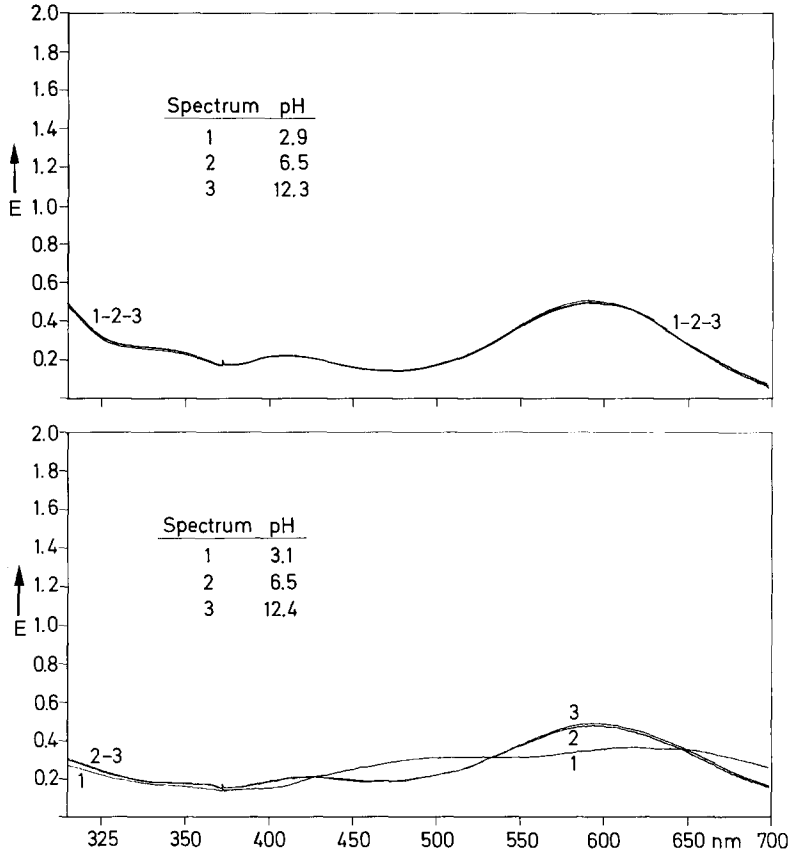

Fig. 6. Absorption spectra of SBB-I and SBB-II at different pH-values, obtained by adding 0.2 ml 0.1 N HCl, 0.2 ml water or 0.2 ml 0.1 N NaOH respectively to a solution of 1 mg dye in 100 ml 70 p.c. ethylalcohol

A further investigation of the pH dependence of SBB-II results in the finding of three isosbestic points upon changing the pH from about 7 to about 1 (Fig. 7). Though because of dissolving problems the spectra have to be measured in ethylalcohol-water (1:1), an estimated equilibriumconstant of the reaction SBB-II + H^+ = SBB-II—H^+ has been determined from the maximal extinction differences at 600 nm at various pH-values. The operational pKa-value amounts to 3.44 indicating that in a alcohol-water mixture (1:1) at pH = 3.44 half of the available dye molecules will be ionised by H^+-ions. So SBB-II behaves as a moderate basic dye, in contrast with SBB-I. The examination of the chromatographic behaviour of SBB-I and SBB-II, both by column and thin layer chromatography across alumina and silica gel revealed, that SBB-I and SBB-II both are retained by these rather polar adsorbents, SBB-I however to a far less extent. Though in the case of preliminary and control experiments, as has been shown in the preceding sections, developing solvents of changing polarity were used, no definite information could be obtained about possible chemical structures, e.g. sidegroups of the

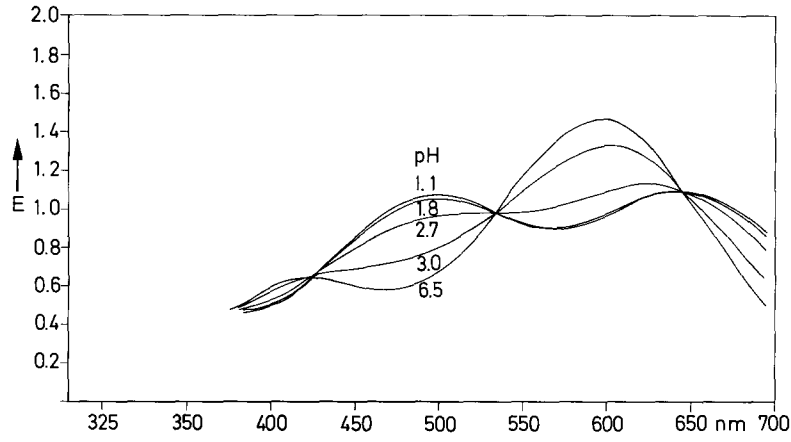

Fig. 7. Isosbestic points of fraction SBB-II: to 8 ml of a 0.0036 p.c. SBB-II solution in 70 p.c. ethylalcohol, in each case 2 ml HCl have been added with normalities of 1.0, 0.1, 0.01, 0.001 and 0 respectively. The spectra have been measured against a blanc alcoholic solution

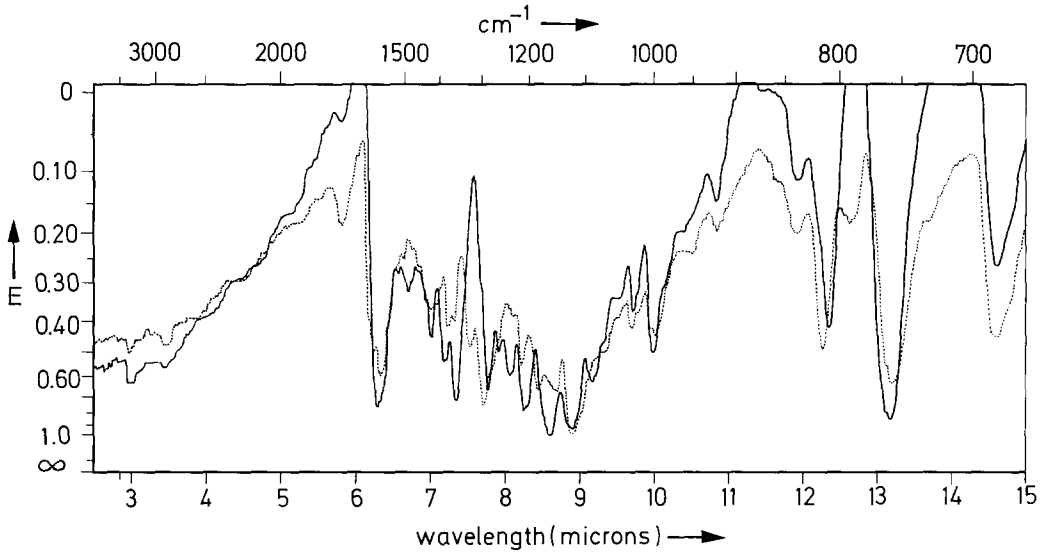

Fig. 8. Infrared spectra of SBB-I (—) and SBB-II (. . .), measured with the Perkin Elmer Infracord on 3 mg dye in about 150 mg solid potassiumbromide

dyes because of the contributions of aromatic rings and azobonds, which at least are certain, to the chromatographic properties.

The main fractions SBB-I and SBB-II therefore were examined by measuring the infrared spectra (Fig. 8). The interpretation of the infrared spectra is rather difficult because of the many peaks in the regio between 1600 and 1000 cm^{-1} due to resonance frequencies of aromatic rings and azobonds. The instability of the dyes may also have some influence on the spectra. Nevertheless the infrared studies yield supporting evidence for the correspondence of the SBB-I and SBB-II structures on the one side and for some marked differences on the other side, as has been shown in Table 5.

Table 5. *Physico-chemical information from the infrared spectra of SBB-I and SBB-II measured in solid potassiumbromide, presented as absorption bands*

Wave-number in cm^{-1}	3,500..... 3,250	1,650... 1,600... 1,550... 1,500...	1,450... 1,400... 1,350... 1,300... 1,250
SBB-I	3,400b	1,620/1,595/80	1,429 1,379/64/45/26 1,299 1,217
SBB-II	3,356	1,600 1,553/27 1,493	1,425 1,389/61 1,284/61/41
Literature			
$-NH_2$	—	———	———
-NH-	.	———	———
Ar-N = N-Ar		— ———	

The position of the peaks (.) and one band (b) has been compared with the position of possible constituents from the literature (RAO, 1963).

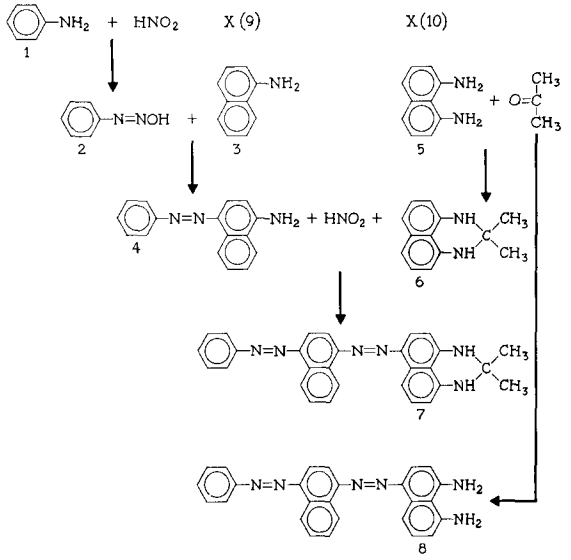

Fig. 9. Scheme of synthesis of Sudan Black B and possible sideproducts according to TERNER (1963). (*7*): "Sudan Black B". (*8*): Other endproducts. (*1*) — (*6*): see text

Indications for primary aminogroups in SBB-I and secondary aminogroups in SBB-II were obtained, in agreement with the larger polarity of SBB-II and with the structure of Sudan Black B and some of its sideproducts (Fig. 9). Nitrogen analysis on the basis of the structural formulas from this figure however has failed to give a definite answer: SBB-I contained 14.35 p.c. nitrogen, and SBB-II 14.60 p.c. nitrogen. The theoretical values on the basis of $C_{26}H_{20}N_6$ (SBB-I ?) and $C_{29}H_{24}N_6$ amount to 20.18 and 18.41 resp. The chemical analysis will be repeated and extended to see whether the structural formulas are correct.

Stability of Sudan Black B, particularly SBB-I and SBB-II

During the present investigation the instable behaviour of Sudan Black B and some of its fractions appeared from the colour change, which became apparent upon standing of solutions during several weeks. Besides the formation of insoluble deposits the colour change from dark blue to violet and brown was an obvious feature.

These instability phenomena took place by the action of light and air, as has been demonstrated by two dimensional thin layer chromatography of commercial Sudan Black B with two different developing solvents (Figs. 10 and 11). If the plate

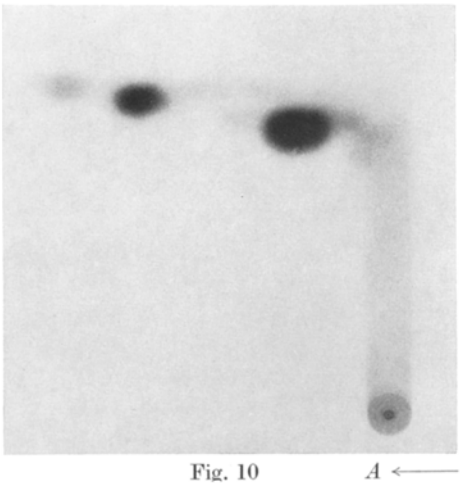

Fig. 10

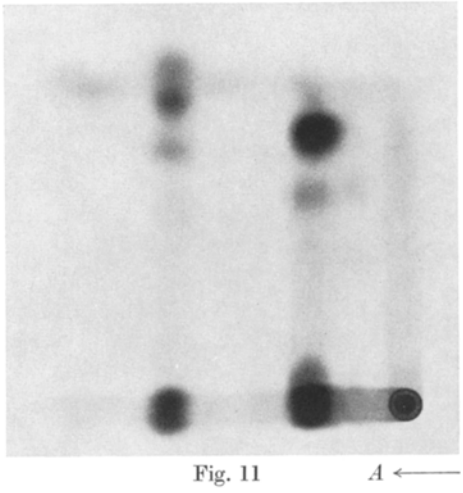

Fig. 11

Fig. 10. Two dimensional thin layer chromatogram of Sudan Black B. Adsorbent: Silica gel G. Developing solvent: First run (*A*): Chloroform — Benzene (1:1), Second run (*B*): Ethylacetate. Between the first and second run the plate was kept in the dark

Fig. 11. Two dimensional thin layer chromatogram of Sudan Black B. Adsorbent: Silica gel G. Developing solvent: First run (*A*): Chloroform — Benzene (1:1). Second run (*B*): Ethylacetate. Between the first and second run the plate was exposed to the action of light and air for 12 hours

was developed in the second eluent in a perpendicular direction, immediately after the first run, all fractions remained homogeneous during the second rum. However when the plate was exposed, after the first run, to the action of light and air for some days, both SBB-I and SBB-II, and also some other primary fractions resolved in more fractions. The appearance of a brown deposit on the starting place particularly agrees with the effects observed after preservation of the dyes (SBB-T, SBB-I and SBB-II) in various solvents. Otherwise SBB-I was less instable than SBB-II.

Our observations agree with those of FREDRICSSON et al. (1958) and KUTT et al. (1959), who showed the effects of ultraviolet light and gases on Sudan Black B. The chemical nature of the degradation or even aggregation products has as yet not been investigated, though FREDRICSSON et al. (1958), studying the influence of acids on Sudan Black B pointed to the decomposition of the pyrimidinenucleus with the formation of the diamine. This mechanism has been questionned by KUTT (1959) by virtue of the appearance of more colours upon the action

of ultraviolet light. Treatment with hydrochloric acid restored to a large extent the blue colour. A further knowledge of the decomposition or aggregation mechanisms would give information about the chemical structure and properties of SBB-I and SBB-II. Meanwhile, the influence on the staining properties is obvious. Therefore the preparation of fresh Sudan Black B solutions and their preservation in dark bottles are important conditions to rule out artefactformation. Also the stained tissue sections have to be preserved in a dark room.

Discussion

Thin Layer Chromatography Compared with other Dye Purification Methods

Thin layer chromatography of commercial Sudan Black B has revealed the presence of a large number of fractions, as in the case of other dyes (Oil Red 0, Sudan III and IV). Though BERMES and MCDONALD (1957) showed ten fractions by column chromatography across celite-silicic acid upon elution with isooctane, our experiments revealed some twenty fractions indicating the larger dissolving power of thin layer chromatography. Repetition of the experiments of SCHOTT (1964), who separated Sudan Black B fractions across a column with alumina and benzene, chloroform-ethanol (1:1) and chloroform as eluents, showed that the obtained yellow, red and blue fractions each contained at least five fractions after thin layer chromatography. Especially the blue fraction of SCHOTT (1964) contained both the fractions SBB-I and SBB-II, the relative amounts being dependent on the elution volume. Even when the alumina column (neutral alumina — Woelm I) was carefully eluted with benzene, chloroform-ethanol, acetone, methanol and alkaline methanol the yellow, red, blue and brown coloured fractions each appeared to consist of more fractions.

Thin layer chromatography of the red powder prepared by SCHOTT (1964) by heating a 1 p.c. aqueous Sudan Black B solution for 8 hours at 60° C and evaporating the filtrate to dryness, resulted in about eight fractions with R_f-values between 0 and 0.25 [silica gel G — chloroform-benzene (1:1)]. The spectral characteristics pointed also to a mixture of Sudan Black B contaminations. Our efforts to obtain more or less pure Sudan Black B fractions by treating alcoholic-aqueous Sudan Black B solutions with hydrochloric acid, sodiumhydroxide or organic acids such as picric acid have also failed. So as yet preparative thin layer chromatography has to be preferred for the purification of Sudan Black B and perhaps of other dyes. From the thin layer chromatography of the Sudan dyes (Fig. 1), otherwise the conclusion has to be drawn, that the polarity of Sudan III, Sudan IV and Oil Red 0 is about the same as SBB-I, the fast migrating Sudan Black B fraction. These four bands show about equal R_f-values in the same developing solvent. Studying the chemical structure of the red dyes, the agreement of the chromatographic behaviour becomes clear, while both Sudan III, Sudan IV and Oil Red 0 are dyes with a 4-aminoazotoluol-azo-b-naphtol structure, with only differences in place and number of methyl groups.

There are no ionisable sidegroups available. Both the chromatographic parameters as the lipid staining of SBB-I correspond with that of the red Sudan dyes. The difference of color, respectively the absorption spectrum needs a larger resonance structure.

The Nature of Sudan Black B

Looking for the nature of Sudan Black B impurities and particularly the difference between the two main fractions, SBB-I and SBB-II, knowledge of the scheme of synthesis is necessary. According to TERNER et al. (1963), (Fig. 9) Sudan Black B (*7*) is synthesized by preparing diazobenzene (*2*) from aniline (*1*), coupling it with α-naphtylamine (*3*) to give 4-benzeneazo-1-naphtylamine (*4*), which is diazotised and coupled to 2.2-dihydro-2.2-dimethylpyridine (*6*). The latter substance is prepared by condensing acetone with 1.8-diaminonaphtalene (*5*).

Sideproducts of the synthesis are possible by incomplete reaction of each step and by the presence of impurities in the starting materials (*9*). Furthermore, incomplete condensation of acetone to diaminonaphtalene and the presence of isomeric diaminonaphtalenes (*10*) give rise to four endproducts (*8*) with the azo-structure of Sudan Black B, but with two free (primary) aminogroups instead of the saturated pyrimidine-ring.

Correlating this information with the results of our chromatographic and spectrophotometric experiments, the nature of the large number of secondary fractions seems certain: starting material and sideproducts of the synthesis of Sudan Black B, of which each step goes incompletely. The blue coloured fractions (X in Table 1) may be the endproducts with nearly the same azo and aromatic structures. As regards SBB-I and SBB-II, which occur for about 20 and 60 per cent of the total commercial dye (this figure being independent on the dye manufacture) both the corresponding and the different properties call attention. Certainly the large similarity of the spectral characteristics point to the correspondence of the chemical structure. However, listing the differences between the main fractions supports the view of at least one large difference.

The R_f-value, by means of which a separation was possible, the effect of pH on the absorption spectra, the histochemical or histophysical properties, the infrared spectra and finally the extinctionquotients E_{425}/E_{475} with their indications for metachromatic or solvatochromic effects, all of these physico-chemical properties pointed into the direction of the easy ionisable dye SBB-II, contrary to the not ionisable or at least less ionisable dye SBB-I.

Returning to the synthesis scheme of Sudan Black B, as published by TERNER (1963) the proposition, that SBB-II has most claim of corresponding with the as yet accepted structure (Fig. 9) of Sudan Black B seems justified, SBB-I possibly being the dye with two primary aminogroups due to the incomplete condensation of acetone to diaminonaphtalene. However this proposition has to be a preliminary one, while the chemical analysis of the purified fractions SBB-I and SBB-II does not support as yet this fact. Whether the disagreeing results of the nitrogen analysis have been caused by the instability of the dyes or by the incorrectness of the structural formulas will be evaluated in a further study, after more infrared spectroscopy and nuclear magnetic resonance studies.

The Influence of Sudan Black B Composition on the Staining Properties

The influence of the chemical composition of commercial Sudan Black B on the staining properties deals with three aspects. First the presence of the basic dye SBB-II to an amount of 60 p.c. causes staining of negatively charged

macromolecules in acid conditions. In case of formalin fixation a pH beneath 5 is easily reached. SBB-II with its positively charged sidegroup(s) particularly will stain nucleic acids, acid mucopolysaccharides and even some proteins, as has been shown by staining of human serum proteins after immuno-electrophoresis with SBB-I, SBB-II and SBB-T respectively: SBB-II and SBB-T both stained albumin, while lipoproteins were also stained by SBB-I.

Secondly, the presence of the large number of secondary fractions to an amount of 20 p.c. may have influence on the staining effect of SBB-I and SBB-II. The yellow, red, green and particularly brown fractions will change the colour obtained. This effect has not been investigated in the present study. However, if the lipid dyes dissolve in the lipids, as has been the most accepted idea, the histochemical reaction is actually a physical one based on partition equilibrium. So each of the components of the dye may have a pronounced influence on the partition and consequently on the colour developed. Moreover, it must be remembered that also the stationary phase viz. the solvent used and the lipid stained by dissolving Sudan Black B influence the partition coefficients. Finally, the instability of both the commercial Sudan Black B dyes as the fractions SBB-I and SBB-II, resulting mostly in brown coloured insoluble deposits, may affect the staining behaviour.

Furthermore, the formation of brown coloured deposits sometimes takes place after staining the tissue sections, again by action of light. As regards the staining properties of the purified fractions, the histochemical part of this study shows, that SBB-I being an almost neutral dye dissolves readily in neutral lipids and gives fairly stable preparations. The basic dye SBB-II also stains neutral lipids, however to a lesser extent in the same time. On the contrary this dye reacts well with phospholipids by virtue of their negative groups. The unspecific staining behaviour of Sudan Black B has to be correlated with the above mentioned aspects, as will be the case with the so called stable sudanophilia (TERNER, 1963), which needs further investigation with purified and homogeneous SBB-fractions.

Solvatochromic and Metachromatic Effects

From the physico-chemical point of view, the boundary between solvatochromy and metachromasia is a gradual one. Solvatochromy is defined as a reversible shift in the absorption spectrum upon changing the solvent. Variations by the chemical interaction of the dye and the coloured substance or variations by pH are classified under metachromasia (HARMS, 1965). So the shift of the absorption spectrum of a dye is due to the interaction with other molecules, whether from the solvent or from the substance investigated: in both cases the resonance structure of the dye will be changed. Moreover, the term "shift" needs some explanation: a shift includes variations of the extinctionvalues, the maxima and minima remaining at the same wavelengths. Therefore in the present study shifts have been characterized by calculation of the ratio of two extinctions at two characteristic points of the spectrum, viz. E_{425}/E_{475}. These extinctionquotients may be taken as a measure for the solvatochromic or metachromatic effect, if the dye obeys the law of Lambert-Beer and the experimental conditions have been standardized.

A qualitative evaluation of the solvatochromic and metachromatic shifts as expressed by the extinctionquotients is possible in the case of pure dyes. At least some information about the lipid stained may be obtained, when each lipid or lipid class shows a characteristic shift of the dye-spectrum. The experiments will be continued to verify this supposition. Otherwise, even staining with commercial Sudan Black B solutions gives information about the lipid stained after some microspectrophotometrical measurements. The lipids of a liver steatosis showed after staining with SBB-T $E_{425}/E_{475} = 1.40$, whereas the phospholipids of myelin showed an extinctionquotient of 1.22 after staining with SBB-T.

So neutral lipids react with SBB-I, and phospholipids with SBB-II, both dyes being supplied in the same mixture. Though these experiments also have to be continued and to be compared by analytical studies, e.g. by histochromatographic studies of the tissue sections, this discussion may be concluded with some remarks concerning the question of the dissolution or the adsorption of the lipid dyes to the lipids. MEIER (1959) and HOLCZINGER (1962) have concluded to adsorption processes, the latter by virtue of the negative temperature coefficient for the dye lipid interaction. Irrespective of the fact that in this consideration the second dye solvent may have a larger temperature coefficient, it seems necessary to repeat these studies with pure Sudan Black B fractions. Perhaps the unpolar dye SBB-I dissolves in the lipid, whereas the more polar or ionised dye SBB-II is adsorbed by chemical interaction with negative groups. In this case SBB-I shows a solvatochromic effect, while SBB-II would give rise to a metachromatic shift. In both cases the accurate estimation of the shift could give information about the lipid stained.

Acknowledgements. The author is grateful to Dr. W. LAARHOVEN and Dr. B. ELLENBROEK (Department of Organic Chemistry) for their helpful advice concerning the organo-analytical work, to Prof. Dr. P. H. M. SCHILLINGS and Dr. J. L. SLOOFF for reviewing this manuscript, and particularly to Miss A. HEUSSEN and Miss A. SCHUELER for their skillful technical assistance.

References

BERMES, E. W., and H. J. MCDONALD: Fractionation and characterization of the lipide stain Sudan Black B. Arch. Biochem. **70**, 49—57 (1957).

CHRISTMAN, J. J., and R. H. TRUBEY: Chromatography and biological stains. I. Paper-chromatography as a means of determining purity and dye content of Sudans III and IV. Stain Technol. **27**, 53—60 (1952).

FREDRICSSON, B., T. C. LEURENT, and B. LÜNING: Decomposition of Sudan Black B causing an artefact in the staining of lipids. Stain Technol. **33**, 155 (1958).

HARMS, H.: Handbuch der Farbstoffe für die Mikroskopie. Lintford: Staufen 1965.

HOLCZINGER, L., and S. BALINT: Experimental data to the theory of fat staining. Histochemie **2**, 389—392 (1962).

JENCKS, W. P., and E. L. DURRUM: Paper electrophoresis as quantitative method: the staining of serum lipoproteins. J. clin. Invest. **34**, 1437 (1955).

KAY, W. W., and R. WHITEHEAD: The role of impurities and mixtures of isomers in the staining of fat by commercial Sudans. J. Path. Bact. **53**, 279 (1941).

KUTT, H., D. LOCKWOOD, and F. MCDOWELL: Decompositions of Sudan Black B by ultraviolett light and gases; its lipid and protein staining properties. Stain Technol. **34**, 203 (1959).

LANSINK, A. G. W.: will be published (1969).

MEIER, W.: Untersuchungen zur Theorie der Fettfärbungen. Z. wiss. Mikr. **64**, 193—208 (1959).

Pearse, A. G. E.: Histochemistry, theoretical and applied. London: Churchill 1960.
Randerath, K.: Dünnschicht-Chromatographie. Weinheim: Verlag Chemie 1962.
Rao, C. N. R.: Chemical applications of infrared spectroscopy. New York: Academic Press 1963.
Schott, H. J.: Zur Frage der histochemischen Fettspezifität des Sudanschwarz B und zur Methodik der Auftrennung seiner Fraktionen. Histochemie **3**, 467—476 (1964).
—, u. W. Schoner: Beitrag zur Fettspezifität des Sudanschwarz B und anderer roter Sudanfarbstoffen bei Reinsubstanzen. Histochemie **5**, 154—162 (1965).
Stahl, E.: Dünnschicht-Chromatographie, ein Laboratoriumshandbuch, 2. Aufl. Berlin-Heidelberg-New York: Springer 1967.
Terner, J. Y.: Stable Sudanophilia: Contributions to the histochemistry of the Sudan dyes. Lab. Invest. **12**, 405—414 (1963).
Trubey, R. H., and J. F. Christman: Chromatography and biological stains. II. Column chromatographic separation of the coloured materials in commercial Sudan III. Stain Technol. **27**, 87—92 (1952).
Werner, H. J., and J. F. Christman: Chromatography and biological stains. III. Comparison of the fat staining efficiency of fractions of commercial Sudan III separated by column chromatography. Stain Technol. **27**, 93—96 (1952).

Dr. A. G. W. Lansink
Department of Pathology, Catholic University
Geert Groteplein Zuid 24
Nijmegen, The Netherlands