The Intracellular Distribution of Fatty Acids in Rat Liver

THE FATTY ACIDS OF INTRACELLULAR COMPARTMENTS

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(*Received* 3 *August* 1960)

A study has been undertaken of the distribution of fats and their constituent fatty acids in the subcellular organelles of the rat-liver cell. A number of investigators have previously studied the fatty acids of rat liver (Kretchmer & Barnum, 1951; Klein & Johnson, 1954; Clement, Clement & Le Breton, 1956; Holman & Widmer, 1959; Dittmer & Hanahan, 1959*a*, *b*), but in all cases the unsaturated fatty acids were measured by ultraviolet spectroscopy or reversed-phase chromatography. These methods do not distinguish individual fatty acids. Gas chromatography (James & Martin, 1952, 1956) of fatty acid esters has made a more complete analysis of fatty acid mixtures possible (see also Paoletti & Grossi, 1960). This technique was used in the studies described in this paper. The results show that although the fatty acids of the nuclei, mitochondria, fluffy layer and microsomes do show differences, the differences are comparatively slight. The layer of supernatant fat, however, shows large differences in fatty acid composition from the rest of the cell fractions and shows similarities in composition to the dietary fat and the adipose tissue. Our results are essentially in agreement with those of Macfarlane, Gray & Wheeldon (1960) obtained in a similar study.

MATERIALS AND METHODS

Animals. These were rats of the Wistar strain, fed on a standard diet of rat cubes supplied by Oxo Ltd. (Medical Department), Southwark Bridge Road, London, S.E. 1. The maker's analysis showed that the cubes contained 3·9% of oil; our analysis showed 3·24% of total lipid extractable by chloroform–methanol and dried at 105° overnight. Loss of some volatile components would be expected under these conditions.

Chemicals. All solvents were of analytical grade except ethanol which was purchased as 'azeotropic'. Diazomethane was prepared from *p*-nitrosomethyltoluenesulphonamide (British Drug Houses Ltd.).

Preparation of the cell fractions. The rats were killed by stunning and decapitation and the livers were transferred to crushed ice. After a few minutes of chilling the livers were passed through the stainless-steel screen of the Fisher mincer (Jouan, Paris) and the pulp produced was weighed. This pulp constitutes the 'liver' for the purpose of this paper. The connective tissue and other material retained by the grid of the mincer (7% of the wet weight) has been ignored. The pulp was homogenized in approx. 2 vol. of ice-cold 0·25M-sucrose with a stainless-steel homogenizer of the Potter–Elvehjem type, and the homogenate was diluted to 20% with 0·25M-sucrose. The procedure for isolating the nuclear fraction and the mitochondria was the same as that of Werkheiser & Bartley (1957), but the fluffy layer was retained and washed in the same way as the mitochondrial sediment. The microsomes were isolated from the combined supernatants of the mitochondrial and fluffy layer fractions by centrifuging for 60 min. at 30 000*g* in the no. 20 rotor of the Spinco centrifuge. The layer of fatty material that floated on the surface of the supernatant was removed and extracted separately.

Extraction of lipids. If distribution studies on lipids are to be meaningful, it is essential either that the extraction of the lipids be quantitative or that the material extracted be a representative sample of the lipid. To assess the completeness of extraction a measured sample of the extract was saponified and the methyl esters of the fatty acids were prepared. The amounts of methyl esters obtained were compared with those obtained by directly saponifying the tissue and preparing the methyl esters. In the best extraction procedure about 90% of the lipid was extracted. Even with the relatively poor extractions the proportions of different fatty acids were the same in directly saponified material as in the saponified extract.

Extraction with chloroform–methanol. Usually the homogenized tissue or tissue fraction was suspended in sufficient 0·25M-sucrose for samples to be pipetted. Samples of liver pulp were weighed directly without homogenization in sucrose. The weighed or measured sample was homogenized in 10 vol. of $CHCl_3$–methanol (2:1, v/v) and kept for 1 hr. After filtering through a fat-free filter paper the solid residue and the filter paper were extracted with a further 10 vol. of the $CHCl_3$–methanol and kept at room temp. for a further 2 hr. The extracts were then combined and subjected to the Folch procedure (Folch, Lees & Sloane-Stanley, 1957). The organic layer was evaporated to dryness and the residue dissolved in $CHCl_3$ containing 2% of methanol. A measured volume of this extract was evaporated to dryness to determine total lipids.

Because of the large volume of the supernatant fraction from the cell fractionation it was impracticable to extract it directly with 10 vol. of $CHCl_3$–methanol mixture. The supernatant was therefore either freeze-dried before extraction with the mixture or the proteins and lipids were precipitated by adding 50% (w/v) trichloroacetic acid to give a final concentration of 10%. The precipitated material

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was washed once with 5% (w/v) trichloroacetic acid and then with water, before extraction with $CHCl_3$–methanol. In one experiment the microsome fraction as well as the supernatant fraction was freeze-dried before lipid extraction and in one experiment all the cell fractions were treated with trichloroacetic acid before extraction. In the latter case the precipitated material was extracted by refluxing in a Soxhlet apparatus for 2 hr. with 100 ml. of $CHCl_3$–methanol (1:1, v/v). The extracted material was evaporated to dryness under a stream of N_2, re-extracted with $CHCl_3$–methanol (2:1, v/v) and subjected to the Folch purification procedure.

Extraction with ethanol–ether. Hanahan, Dittmer & Warashina (1957) have used extraction with ethanol followed by ethanol–ether mixtures to remove tissue lipids. In one experiment their procedure was followed and tissue fractions were extracted with 10 vol. of 95% ethanol at room temp. for 4 hr. The residue was then re-extracted with 10 vol. of ethanol–ether (3:1, v/v) for a further 3 hr. The latter extraction was repeated and the three extracts were combined. After the extracts were dried, the residue was exhaustively extracted with warm light petroleum (b.p. 40–60°). A sample of this extract was dried for determination of total lipid.

Direct saponification. A measured portion of the tissue, tissue homogenate, or tissue fraction (equivalent to not more than 1 g. wet wt. of tissue) was incubated for 3 days at 30° with 5 ml. of methanolic KOH containing quinol (20 ml. of 10N-KOH, 50 ml. of methanol, 0·1 g. of quinol, 30 ml. of water).

Saponification of the lipid extracts. A measured volume of the lipid extract was evaporated to dryness and 5 ml. of saponification mixture was added. The mixture was then kept at 30° for 3 days.

Extraction of the fatty acids. Water (15 ml.) was added to the saponification mixture and then 15 ml. of light petroleum (b.p. 40–60°). After thorough shaking, the solvent layers were allowed to separate and the upper phase was drawn off with a pipette. The extraction was repeated twice with 15 ml. portions of light petroleum. The water layer was then acidified by the addition of 2 ml. of 50% (v/v) H_2SO_4 and extracted three times with 15 ml. portions of the light petroleum.

Preparation of the methyl esters of the fatty acids. Freshly prepared diazomethane in ether was added to the chilled petroleum extracts of the fatty acids [dried by the addition of a mixture of anhydrous Na_2SO_4 and anhydrous $NaHCO_3$ (4:1, w/w)] until the solution remained permanently yellow. After 30 min. the solution was warmed to remove excess of diazomethane and stored at 0° until required for gas chromatography or estimation of methyl esters. In the esterification with methanol the esters were made and purified according to Stoffel, Chu & Ahrens (1959). In all cases the organic solvent was removed from the methyl esters by evaporation under a stream of N_2.

Gas chromatography. All samples were chromatographed on two different columns. Two chromatographs were used simultaneously. One was a Pye Argon chromatograph (Pye Ltd., Cambridge) and the other similar apparatus was built in this laboratory with the assistance of Mr A. Renshaw. The design for column and detector, which used a 10 ml. strontium foil, was kindly provided by Dr J. E. Lovelock (see Lovelock, 1958). The amplifier was a simple, single-valve (Osram ET/3) amplifier kindly supplied by Mr A. Thomson of the Dyson Perrins Laboratory, Oxford. The recorder was a Honeywell Brown 2 mv full-scale potentiometric recorder. The Pye Argon chromatograph housed a column packed with 20% Apiezon L on Embacel (May and Baker Ltd.) sieved to 90–100 mesh. The argon flow rate was 50 ml./min. and the temp. (200°) was maintained by constant voltage heating. This column gave good recoveries of standard mixtures, which included methyl linoleate (California Foundation) and was used as the reference column throughout this study. The other machine housed a column 4 ft. long and 4 mm. in internal diameter, packed with acid-washed Celite (100–120 mesh) coated with 15% polyethylene glycol adipate ester. The ester was prepared, on the advice of Dr A. T. James, by heating together ethylene glycol and adipic acid in approximately equimolar quantities for 2 hr. at 180° in the presence of a small quantity of *p*-toluenesulphonic acid as a catalyst. The column-packing material, after coating, was resieved to 100–120 mesh before packing. The column was preheated in a stream of argon for about 72 hr. before use for analysis and was operated at 175° with a flow rate of argon of about 60 ml./min. The complementary data from chromatographic analyses on these two columns were combined by using ratios of single components common to both. Major acids were identified by comparison with the retention times of pure standards, all times being measured from the air peak. Some other acids were tentatively identified by comparison of their retention times, relative to methyl stearate, with those published in the literature, e.g. Insull & Ahrens (1959). Where the two chromatograms were in disagreement, as they occasionally were for the unsaturated esters, reliance was placed on the results of the Apiezon column, since the polyester columns sometimes underestimate these acids (Orr & Callen, 1959). The exception to this is methyl oleate which was sometimes difficult to assess accurately on the Apiezon column owing to its poor resolution from methyl linoleate. The fatty acid notation is that suggested by Ahrens *et al.* (1959). Thus oleic acid is designated 18–1 and arachidonic acid 20–4, indicating the numbers of double bonds without indicating their position.

Determination of dry weight of tissues. Samples of the tissues, homogenates, or tissue fractions were treated with 10% (w/v) trichloroacetic acid. The precipitated protein and lipid were treated according to Werkheiser & Bartley (1957).

Measurement of fatty acid esters. This was according to Rapport & Alonzo (1955).

RESULTS

Efficiency of the extraction procedures

When extractions of lipids by three different methods (by chloroform–methanol, ethanol–ether and chloroform–methanol after trichloroacetic acid treatment) from liver pulp were compared large differences were found. Because of the variation, a more detailed investigation of lipid extraction from liver pulp and mitochondria was made. Table 1 compares the amounts of 'lipid' extracted directly from the tissue with that extracted from the tissue homogenized in 0·25 M-sucrose. Whereas there is no difference in the amount of 'lipid' extracted when chloroform–methanol was used

Table 1. *Extraction of lipids from liver pulp and mitochondria*

For extraction procedures see text.

Tissue fraction	Extraction method	Weight of 'lipid' extracted/g. of wet tissue or/ml. of mitochondrial suspension (g.)	Methyl esters formed after extraction and saponification (μmoles/g. dry wt.)
Rat-liver pulp	Chloroform–methanol	0·0406	63·0
Rat liver homogenized in 0·25 M-sucrose	Chloroform–methanol	0·0411	63·4
Rat-liver pulp	Ethanol–ether	0·0576	86·0
Rat liver homogenized in 0·25 M-sucrose	Ethanol–ether	0·0816	85·0
Rat-liver mitochondria	Chloroform–methanol	0·0063	6·7
Rat-liver mitochondria	Ethanol–ether	0·0072	8·9

Table 2. *Comparison of percentage composition of fatty acids of liver homogenates as shown by direct saponification of the tissue and by saponification after extraction with chloroform–methanol* (2:1)

For details of extraction procedure see text. The percentages are calculated on a molar basis. br., Branched chain.

Fatty acid No. of C atoms and double bonds	Percentage of fatty acid present after: Direct saponification	Extraction
14–0 (myristic)	0·64	0·68
15–0	0·63	0·61
16–0 (palmitic)	19·1	21·6
16–1 (palmitoleic)	Present	Present
17–0 (br.)	Present	Present
18–0 (stearic)	19·1	17·7
18–1 (oleic)	19·7	19·1
18–2 (linoleic)	24·3	24·0
20–4 (arachidonic)	13·6	13·4
22–6	3·25	3·09
Unidentified compound	3·64	3·51

Table 3. *Total lipid content of rat liver and rat-liver subcellular fractions*

Mean values for total lipids extractable by exhaustive extraction.

Fraction	Lipid as percentage of dry wt. of tissue fraction	Fatty acid esters/g. dry wt. of tissue fraction (μmoles)
Liver pulp	23	363
Nuclei	15·8	265
Mitochondria	23·4	384
Fluffy layer	24·3	464
Microsomes	30·9	525
Supernatant	11·2	106

there was, apparently, an increased extraction from the homogenate in sucrose when ethanol–ether was used. This increase was shown to be partly due to extraction of sucrose.

The ethanol–ether extractions did, however, extract appreciably more lipid than the chloroform–methanol as shown by the larger amounts of the methyl esters of the fatty acids that could be prepared from extracts made with ethanol–ether. Similar results were obtained with mitochondria. The differences could be up to 25% of the lipid found. Usually about 90% of the esters shown to be present by the direct saponification technique could be recovered in the extracted lipids when either chloroform–methanol or ethanol–ether was used for extraction. Whereas the precipitation of the lipids of the supernatant fraction did not result in low recoveries, trichloroacetic acid treatment of the particulate fraction or freeze-drying of the sucrose suspension before extraction resulted in recoveries as low as 60%.

Some 5–10% of the lipid of rat liver is very resistant to extraction by the established procedures, but despite variability in the amount extracted there was no indication that lipids extracted did not constitute a representative sample. This is illustrated in Table 2. Similar observations were made on the isolated subcellular fractions. Sex, age or weight of the rats had no obvious effect on the composition of the fatty acid mixture extracted from the liver.

Total lipids of the cell fractions

From the preceding section it is clear that the amount of lipid found in a tissue will depend on the extracting procedure. In Table 3 are given the mean values for the total lipids extractable from the tissue or tissue fraction by exhaustive extraction with chloroform–methanol or ethanol–ether. Of the cell organelles the microsomes contained the highest and the nuclei the lowest proportions of fat.

Changes in the fatty acid composition of homogenates during incubation

Table 4 shows that there was no appreciable change in the fatty acid composition of a rat-liver homogenate incubated for 2 hr. at 37°. It is unlikely, therefore, that the composition of the fatty acids would have changed much in the tissue fractions during the time taken to isolate them from the homogenate. It is, however, possible that

some redistribution of fatty acids may have occurred either between the lipids or the cell organelles.

Fatty acids of the cell fractions

Table 5 presents the mean fatty acid composition of the whole tissue and its constituent cell fractions. The acids in the table comprise more than 95% of the total acids found (trace acids are omitted). No marked differences were seen between the fatty acid distributions in the cell fractions except in the fatty supernatant layer. Of the particulate fractions the mitochondria have the highest concentration of polyunsaturated acids and the lowest oleic and palmitic acid content.

Comparison of the fatty acid composition of the diet, the adipose tissue and the fatty supernatant

Table 6 shows a comparison of the fatty acid composition of the fatty supernatant with that of the dietary fat (rat cubes) and the rat inguinal adipose tissue. They all exhibit low stearate and high oleic and linoleic acid contents. They also have significant amounts of linolenic acid which is present in the diet in rather higher concentration. On the other hand, the fatty supernatant has much higher content of polyunsaturated acids, arachidonic (20–4) and docosahexaenoic (22–6), than the diet and the adipose tissue. In this respect, it is closer to the composition of the rat-liver homogenate. Adipose tissue fat is predominantly triglyceride in nature, and so is that of the diet.

Table 4. *Comparison of percentage fatty acid composition of liver homogenate before and after incubation*

Extraction with chloroform–methanol (see text). The percentages are calculated on a molar basis.

No. of C atoms and double bonds	Homogenate	Homogenate after 2 hr. incubation
14–0 (myristic)	0·82	0·86
15–0	0·81	0·80
16–0 (palmitic)	28·3	28·0
16–1 (palmitoleic)	2·63	2·38
17–br.	—	0·84
17–0	1·14	0·77
18–0 (stearic)	16·0	16·8
18–1 (oleic)	19·5	20·3
18–2 (linoleic)	15·9	14·9
20–4	11·7	10·3
20–3	—	Present
22–6	3·34	4·18

DISCUSSION

The similarity of particulate fatty acid composition shown in this work is of interest. If particle-bound fat is related to the activities of the individual cell particles, it is reasonable to expect the fat to be more specific to the particular organelle, and to reflect in its composition the differences which are well known to exist in, for example, microsomal and mitochondrial function. Both Clement *et al.* (1956), using rats, and Kretchmer & Barnum (1951), using mice, showed larger differences between the fatty acid content in the different organelles. Both these groups of workers found a higher content of arachidonic than of linoleic acid in the mitochondria and microsomes, the reverse of what we have observed in the present study. The former workers showed a higher content of unsaturated acids in the microsomes than mitochondria, whereas Kretchmer & Barnum also found, as we have done, that the mitochondrial fatty acids are the more highly unsaturated. In both these former studies, diets different from ours may have been used, and the animals used were different.

In one of the groups of rats used by Klein & Johnson (1954) very large concentrations of triene

Table 5. *Mean percentage composition of fatty acid of rat liver and rat-liver subcellular fractions*

The numbers in parentheses are the numbers of experiments. Fatty acids present in trace quantities only are not given. The percentages are calculated on a molar basis.

No. of C atoms and double bonds	Pulp (4)	Nuclei (4)	Mitochondria (5)	Fluffy (3)	Microsomes (4)	Super-natant (4)	Fatty supernatant (2)
14–0 (myristic)	0·70	0·50	0·41	0·44	0·47	0·79	1·41
15–0	0·65	0·57	0·47	0·59	0·53	0·68	0·78
16–0 (palmitic)	24·6	25·7	21·4	21·7	23·4	22·7	24·2
16–1 (palmitoleic)	2·46	1·66	1·63	1·25	1·27	2·34	4·19
17–0	0·83	0·64	0·74	0·84	1·01	0·74	0·31
18–0 (stearic)	13·5	16·4	17·5	17·1	19·9	13·2	1·81
18–1 (oleic)	16·1	14·0	12·2	13·2	13·7	21·6	29·5
18–2 (linoleic)	20·2	16·5	20·6	17·7	16·0	18·7	28·1
20–3	1·07	0·92	1·84	1·10	1·30	0·89	0·27
20–4 (arachidonic)	11·9	14·1	15·4	16·3	15·4	10·3	2·03
22–5	0·90	0·64	0·77	0·93	0·78	0·46	0·95
22–6	4·33	5·96	5·12	5·15	4·73	2·46	2·04
Total unsaturated acids	57·0	53·8	57·5	55·6	53·1	56·8	67·1
Polyunsaturated acids	38·4	38·1	43·7	41·2	38·2	32·9	33·4

Table 6. *Comparison of the percentage fatty acid composition of the dietary fat with that of the adipose tissue, the 'floating supernatant' and the liver homogenate*

Extraction with chloroform–methanol (see text). The percentages are calculated on a molar basis.

No. of C atoms and double bonds	Food: rat cubes (5 g.)	Rat inguinal adipose tissue (single rat)	Floating fatty supernatant (pooled from 8 rats)	Rat-liver homogenate (single rat)
9–br.	—	0·14	—	0·05
12–0 (lauric)	—	0·056	0·060	0·06
13–0	—	—	—	0·08
13–br.	—	—	—	—
14–0 (myristic)	0·42	0·99	1·1	0·82
14–1 (myristoleic)	—	—	—	0·04
15–0	—	0·296	0·56	0·50
15–br.	—	0·081	—	0·21
16–0 (palmitic)	18·2	23·2	26·4	25·00
16–1 (palmitoleic)	1·0	3·00	2·86	1·9
16–br.	0·32	0·42	—	—
17–0	—	0·368	0·39	0·25
17–br.	—	0·217	0·31	—
18–0 (stearic)	1·59	2·89	2·02	11·9
18–1 (oleic)	29·9	35·2	28·1	20·5
18–2 (linoleic)	40·5	31·2	27·1	22·8
18–3 (linolenic)	3·1	0·290	0·56	—
19–br.	—	0·256	0·66	—
19–0	1·2	—	—	—
20–0	—	—	0·55	—
20–1	1·25	0·678	0·76	0·30
20–2	—	—	0·45	—
20–3	0·61	—	0·55	0·20
20–4 (arachidonic)	0·86	0·617	1·98	9·80
20–5	—	—	0·51	0·54
21 unsaturated	—	—	0·83	—
22–6	1·07	0·119	3·13	3·71
22–5	—	0·052	1·39	0·50
24 unsaturated	—	—	0·56	0·67
Total unsaturated acids	78·29	71·16	68·78	60·92
Polyunsaturated acids	46·18	32·28	35·11	37·55

acids appeared in the older animals, which they thought to be due to the diet being marginally deficient in essential fatty acids. The concentration of eicosatrienes (20–3) was not remarkably high in any of our analyses.

The arachidonate/linoleate ratio is highest in the microsomes in this study and it has been established that linoleate gives rise to arachidonate *in vivo* (Steinberg, Slaton, Howton & Mead, 1956). This suggests the possibility that the microsome enzymes may be involved in the transformation of linoleate to arachidonate.

The fatty acid composition of the fluffy layer as compared with that of mitochondria and microsomes is consistent with the conclusion, but does not by any means prove, that the fluffy layer is a mixture of mitochondria and microsomes.

SUMMARY

1. The fatty acid composition of rat-liver homogenates and subcellular fractions prepared by differential centrifuging has been measured.

2. Complete extraction of all lipid material from the liver was not achieved by the standard extraction procedures. The proportions of individual fatty acids in the extracted lipids were the same irrespective of the percentage of lipid extracted (60–90%). The fatty acids liberated by direct saponification of the tissue were in the same proportions as in the extracted lipids.

3. The proportions of fatty acids in a rat-liver homogenate remained constant during a 2 hr. aerobic incubation at 37°.

4. The mean fatty acid content (expressed as μmoles of fatty acid/g. dry wt. of tissue) were: liver pulp 363, nuclei 265, mitochondria 384, fluffy layer 464, microsomes 525, supernatant 106.

5. The fatty acid compositions of the different subcellular fractions were very similar. The main fatty acids were palmitic, stearic, oleic, linoleic, arachidonic and docosahexaenoic acid. Mitochondria contained the highest proportion of polyunsaturated fatty acids (43·6%).

6. The fatty acid composition of the supernatant fat after removal of particulate material was similar to that of the food, and similar to that of the adipose tissue.

7. The implications of the results are discussed.

We wish to thank Professor Sir Hans Krebs, F.R.S., for his interest and advice, Mr A. Renshaw for his assistance in building a gas chromatograph, Dr Lovelock and Dr James for prepublication advice on the techniques of gas chromatography, and Miss B. M. Notton for technical assistance. The work was aided by grants from the Rockefeller Foundation and the United States Department of Public Health. One of us, G.S.G., held a Nuffield Demonstratorship during the period of the research, and wishes to thank the Nuffield Dominions Trust for this financial support.

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Determination of Inorganic Sulphate in Studies on the Enzymic and Non-Enzymic Hydrolysis of Carbohydrate and Other Sulphate Esters

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(*Received* 21 *July* 1960)

During studies on the hydrolysis of potassium glucose 6-*O*-sulphate by hydrazine (see Dodgson & Lloyd, 1961) it became necessary to develop a new method for determining small amounts of inorganic sulphate. Previous methods developed in these laboratories have involved the use of benzidine (Dodgson & Spencer, 1953; Spencer, 1960) or barium chloranilate (Lloyd, 1959) but cannot be used in the presence of hydrazine. In a previous study of the non-enzymic hydrolysis of crude samples of glucose monosulphate (Dodgson & Spencer, 1954), liberated sulphate was estimated gravimetrically as barium sulphate, but the method was not satisfactory because of the relatively large amounts of substrate which had to be used.

The author's attention was directed (Dr B. Sörbo, personal communication) to the possible use of a turbidimetric method for the determination of inorganic sulphate, gelatin being used as a cloud stabilizer. A method of this type, based on that of Gassner & Friedel (1956), has been developed by Berglund & Sörbo (1959) for the determination of inorganic sulphate in blood and urine. The present paper shows how the method was adapted to the study of the non-enzymic hydrolysis of simple carbohydrate sulphates. Other modifications are described which enable the method to be used, under certain circumstances, for the assay of glycosulphatase and for the microanalysis of ester sulphates.

MATERIALS

Trichloroacetic acid. This was British Drug Houses Ltd. AnalaR reagent.

Barium chloride–gelatin reagent. Micromethods which depend on precipitation of SO_4^{2-} ions in one form or another generally require a certain minimum amount of