METABOLISM AND FUNCTIONS OF PHOSPHATIDES

SPECIFIC FATTY ACID COMPOSITION OF THE RED BLOOD CELL MEMBRANES

F. KÖGL*, J. DE GIER, I. MULDER AND L. L. M. VAN DEENEN
Laboratory of Organic Chemistry, State University of Utrecht (The Netherlands)
(Received January 26th, 1960)

SUMMARY

In attempting to establish a specific lipid composition of biological interfaces, the fatty acid composition of the lipid moiety of the red cell membrane of a number of mammals was determined by means of gas–liquid chromatography. A rather constant and characteristic fatty acid pattern proved to occur in the membranes of the red cells of each species. Quantitatively striking differences were found between the fatty acid compositions of the red cell membranes of the various mammals. Most pronounced were differences in the palmitic acid and oleic acid content. A gradual decrease in the ratio palmitic acid–oleic acid was shown in the red cell membranes of: rat, man, rabbit, pig, horse, ox and sheep in that order. Since this arrangement rather resembled the sequence based on permeability properties of these membranes taken from the literature, a possible relationship between fatty acid composition and permeability of membranes is discussed.

INTRODUCTION

The obvious differences in permeability of the numerous interfaces which regulate the transport between cells and their environment, and also between the cell particles inside the cell, suggest a specific frame of each of these various biological membranes. Theories attempting to formulate the arrangement of molecules in the biological membranes, all recognize the importance of complexes formed by an association of proteins with lipids[1–4]. Divergencies in the properties of the interfaces may be dependent on the orientation, the types, and the ratio of the molecules or molecular fragments, participating in the various chemical and physico-chemical combinations. Whereas the variability of the protein moiety of the corresponding lipoproteins appears theoretically to be nearly unlimited, the variation of the lipid part, consisting mainly of cholesterol and phosphatides, seems to be more restricted. Differences in the fatty acids of the lipid molecules, however, may also contribute to a distinction between the various biomembranes. The chain length and the degree of saturation of the fatty acid constituents may play a part in the functional mechanism of the

* Deceased June 6th, 1959.

biological interfaces. Hence the question arose, whether different biomembranes are characterized by a specific fatty acid composition.

A difficulty in studying membrane lipids is to isolate them specifically from the tissues, without contamination with other lipids. Of course this difficulty disappears if one can isolate the membranes of cells or cell particles. This seems to be possible with mammalian red cells, where, by osmotic means, posthemolytic residues ("ghosts") may be obtained that should represent the plasma membrane as closely as possible. It was therefore decided to make comparative analyses of the fatty acid composition of ghost lipids of red cells obtained from rat, rabbit, pig, sheep, ox, horse, and of man.

EXPERIMENTAL

Preparation of ghosts

Erythrocytes were gained by centrifugation of whole blood, previously defibrinated by shaking with glass beads. The cells were washed 3 times with 0.9 % NaCl solution at room temperature. After hemolysis for 18 h in 10 volumes of water saturated with CO_2, at 3°, ghosts were prepared according to the CO_2 method of PARPART[5], omitting, however, the saline washings which were suspected to give a loss of wanted material. The yield of posthemolytic residues recovered from the cells of different species after lyophilization varied from about 3 to 5 g/l of whole blood.

Extraction of the lipids

Lipids were extracted by treating about 4 g of dry ghosts with 500 ml of ethanol–ether (3:1; v/v) at room temperature for 18 h. The extraction was repeated twice in a similar way, but now for 1 h. The combined extracts were evaporated *in vacuo* under nitrogen, leaving 0.8–1.2 g of membrane lipids. These lipids were stored *in vacuo* in the dark at 2°.

Saponification

Saponification was carried out by refluxing about 250 mg of the extracted lipids with 10 ml of a *N* NaOH solution in ethanol in a nitrogen atmosphere for 2 h. After diluting with water unsaponifiable material was removed by extraction with ether, freed from peroxides as usual. The resulting aqueous soap solution was acidified with sulfuric acid and the fatty acids were extracted with ether.

Esterifying

After drying the etheric solution with anhydrous Na_2SO_4 the fatty acids were esterified with a diazomethane solution in ether. The solution of fatty acid methyl esters was filtered and evaporated *in vacuo*.

Gas–liquid chromatography

The fatty acid methyl esters, diluted some 5 times with ether, were analysed by means of gas chromatography. A Perkin-Elmer 154 B Vapor Fractometer was used, supplied with an electronic temperature regulator (as in Perkin-Elmer 154 C) so as to obtain a good base line at high temperatures. Condensation of products before they reached the column caused a very bad tailing on the chromatograms. This was

remedied by additional heatings both of the injection block and of the tube connecting the injection block with the column. Also the venting tube had to be warmed up electrically to prevent condensation of the vapours as they left the column. As the thermistor katharometer has no high sensitivity at the used high temperatures, and as large samples had a bad effect on the separation especially on column b (see below), the sensitivity of the used 5 mV Leeds and Northrup Speedomax recorder was raised 3.5 times. Furthermore, this recorder was modified so as to come to a complementary chart speed, one sixth of the normal chart speed of 1 cm/min.

Two different columns were used:

Column a: Silicon grease stationary phase. Dow Corning high vacuum silicon grease was purified by solving it in ethyl acetate and precipitating with ethanol[6]. The solid support was an acid washed, coarse fraction of Celite 545 (*cf.* ref. 7). The inner diameter of the 2-m stainless steel column was 4½ mm. The working temperature was 225° and the flow 50 ml of He/min.

Column b: Diethylene glycol succinate stationary phase. Poly diethylene glycol succinate solution was obtained from Research Specialties Co. The solvent was evaporated and any low boiling material was eliminated by heating to 200° *in vacuo*. The solid support consisted of 80–100 mesh Celite, washed with acid and alkali as described by LIPSKY *et al.*[8]. Use was made of two one meter columns of pyrex glass with an inner diameter of 4½ mm, in series. The working temperature was 200°, the flow 105 ml of He/min.

In both cases the inlet pressure was 30 lbs/in^2, the outlet atmospheric. The stationary phases (1 part) were solved for column a in ether and for column b in acetone and mixed with their respective solid supports (3 parts). The solvent was evaporated *in vacuo* with frequent shaking and heating up to 100°.

With column a the methyl arachidate peak appears within 75 min, with column b within 15 min. As a rule for column a chromatograms the recorder was used at normal sensitivity and low chart speed, for column b chromatograms at high sensitivity and high chart speed. The separation by column b was affected much more by too large sample volumes than the separation by column a*.

Samples of 5–50 μl of the methyl ester solutions in ether were put onto the column by means of a 250-μl hypodermic syringe.

RESULTS

Qualitatively some, or all, of the following fatty acid constituents are found in the lipid samples from red cell membranes: lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and arachidonic acids. No arachidic acid was found. Identification was carried out by comparing the retention times on the two different columns with the retention times of the members of known mixtures of fatty acid

* Also other columns were tried with the following stationary phases: laboratory-made poly diethylene glycol succinate, poly diethylene glycol adipate (LAC-1-R 296, Cambridge Inds. Co., and Reoplex 400, Geigy, respectively) and Apiezon N, and furthermore the columns A, B and P as obtained from Perkin-Elmer Co. However, the described combination of columns a and b showed to be the most satisfactory. Moreover, column a was chosen since it was nearly undestroyable: after two years of use there is no appreciable loss of separation, and retention times are only slightly diminished. Furthermore, column b gave the most rapid analysis of our esters, and the separation was not even less than with column P of Perkin-Elmer Co.

methyl esters. This identification was enhanced by the fact that with column a the saturated esters appear next to the unsaturated ones with the same chain length, the latter without adequate separation from one another, whereas with column b the saturated esters appear first and the unsaturated ones are very well separated. Moreover, hydrogenation of the fatty acid methyl esters with the aid of Pt catalyst and subsequent rechromatographing gave additional information. Another confirmation of our identifications was obtained by reversed phase paper chromatography of the free fatty acids*.

Quantitative determinations of the fatty acid composition of our lipid samples were carried out by measuring peak areas on the gas chromatograms. For this purpose mainly chromatograms with column b were used because of the better separation of all components of our samples on this column. Areas were determined by multiplying the peak height by the width at half height. Analysis of weighed amounts of standard mixtures of fatty acid methyl esters showed peak areas to be directly proportional to component weights with the same proportionality factor for each ester from laurate to arachidate, so peak area percentage of total measured peak areas is identical with weight percentage of fatty acid methyl ester in the sample.

In Fig. 1 some representative gas chromatograms are shown. The quantitative results of all of our experiments are summarized in Table I.

In the second column of this table the number of animals is given from which blood was pooled before the analysis. The figures in the following columns represent

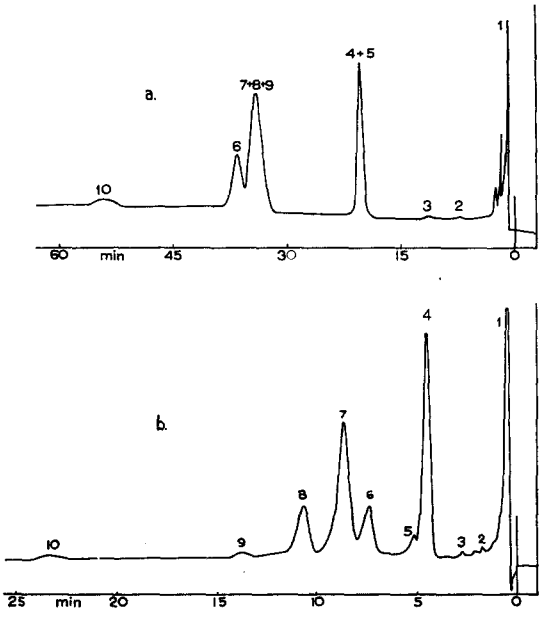

Fig. 1. Examples of actual gas chromatograms. a, fatty acid methyl esters from pig ghost lipids on column a; b, same on column b. For details see text. 1, ether; 2, laurate; 3, myristate; 4, palmitate; 5, palmitoleate; 6, stearate; 7, oleate; 8, linoleate; 9, linolenate; 10, arachidonate.

* Whatman No. 1 paper was impregnated with petroleum, according to WAGNER *et al.*[9]. The fatty acids were stained by the Cu dithiooxamide method, and iodine vapour revealed only the unsaturated ones.

TABLE I

PERCENTAGES OF FATTY ACIDS IN GHOST LIPIDS

Percentages are given of total measured peak areas on gas chromatograms of column b of the methyl esters from laurate to linolenate. + denotes that the peak is too small for accurate measurement. The standard errors of the means* are placed between brackets.

Animal	*Number of animals*	*Laurate*	*Myristate*	*Palmitate*	*Palmitoleate*	*Stearate*	*Oleate*	*Linoleate*	*Linolenate*
Rat	17	+	+	45	—	21.5	17.5	15.5	0.5
Rat	8	+	1	41	2	23.5	18.5	14	—
Mean		+	0.5 (0.5)	44 (1.9)	0.5 (1.0)	22 (1.0)	18 (0.5)	15 (0.7)	+
Man (A, rh. pos.)	1	+	+	40.5	2.5	15	25	16	1
Man (A, rh. pos.)	3	+	—	38.5	2.5	15	27	17	+
Man (B, rh. pos.)	1	+	+	37.5	2	13	25.5	20	2
Man (B, rh. neg.)	1	—	+	37	—	14.5	25.5	20	3
Man (O, rh. pos.)	2	+	+	38.5	—	16.5	29	14	2
Man (O, rh. pos.)	2	+	+	34	+	16.5	28.5	19	2
Man (O, rh. pos.)	3	—	+	36	2	15	25.5	18	3.5
Man (O, rh. pos.)	1	—	+	39.5	2	17.5	26.5	13.5	1
Mean		+	+	37.5 (0.7)	1.5 (0.4)	15.5 (0.5)	26.5 (0.6)	17 (0.8)	2 (0.5)
Rabbit	5	—	—	40	5	10.5	28.5	16	—
Rabbit	4	1	0.5	33	4.5	11.5	24	23.5	2
Rabbit	7	+	+	34	3	11	23	28	1
Mean		+	+	36 (2.1)	4 (0.7)	11 (0.3)	25 (1.6)	23 (3.7)	1 (0.6)
Pig	1	—	+	29.5	2.5	10	38	20	—
Pig	1	+	0.5	31	3	17	31	14	3.5
Pig	3	+	+	32.5	1.5	14	36.5	14.5	1
Pig	2	+	+	26	1.5	16	33.5	22	1
Mean		+	+	30 (1.6)	2 (0.4)	14.5 (1.3)	35 (1.3)	17.5 (2.0)	1 (0.6)
Horse	1	+	+	25.5	4	19	30.5	19.5	1.5
Horse	2	+	+	25.5	4	18.5	34	18	—
Horse	3	+	+	18.5	1	11.5	26	40.5	2.5
Horse	2	+	+	19	2.5	12.5	32.5	30	3.5
Mean		+	+	21.5 (1.9)	2.5 (0.8)	14.5 (1.9)	30 (2.0)	29.5 (5.5)	2 (0.8)
Ox	1	—	+	21.5	3.5	17	47	10.5	+
Ox	3	—	+	11	3	14	53	15.5	3.5
Ox	2	—	+	12	4.5	14	52	16	1.5
Mean		—	+	13 (2.7)	3.5 (0.5)	14.5 (0.8)	52 (1.6)	15 (1.4)	2 (1.0)
Sheep	1	1.5	1	17	3.5	10	55	10	2.5
Sheep	3	—	+	10	3.5	8	63.5	8.5	7
Sheep	3	+	—	13.5	2	6	62	11	5.5
Mean		+	+	12.5 (1.7)	3 (0.6)	7.5 (1.0)	61.5 (2.0)	10 (0.9)	5.5 (1.1)

* The mean values have been calculated taking into account the number of animals of each experiment. This procedure was justified by the fact that the standard deviation of analyses of one sample of blood which was done in quadruplicate, was significantly lower than the standard deviation of experiments of different samples of blood.

weight percentages of the methyl esters found. Data on the amount of arachidonic acid are not given, for not all of our chromatograms were continued until arachidonic acid methyl ester left the column. However, some of the samples studied, mainly of the animals first mentioned in the table, contained considerable amounts of this acid. Fatty acids of higher unsaturation or with a longer carbon chain were neglected.

Examination of the table indicates that in all studied species the fatty acid composition of the erythrocyte membrane reveals for a certain species a strikingly characteristic and constant pattern. Although in most cases it was not possible to establish the origins and antecedents of the animals, no marked differences were noticed in the analyses carried out in various seasons, with involved differences in the diets of the animals. Although we worked up our samples under nitrogen, incidentally some oxydation may have taken place of linoleic and linolenic acids. Probably this does not explain the variability in the amount of linoleic acid obtained in the analyses of horse ghost lipids. However, we know that only the horses of the third experiment, giving the highest amount of linoleate, were grown up in East Europe.

Moreover, not only a rather constant fatty acid composition of the erythrocyte membrane of one species appears to exist, but pronounced differences are also noted in this respect between the various species. The red cell membranes of the series rat, man, rabbit, pig, horse, ox and sheep show a gradual decrease of the palmitic acid concentration. A corresponding shift in the oleic acid amount—but in opposite direction—proved to occur in the same order, with a low percentage of oleic acid in the red cell membrane of the rat, up to an extremely high amount in the sheep.

DISCUSSION

The results of our experiments indicate a specific fatty acid composition of the lipid moiety of the red cell membrane, which is qualitatively almost the same, but quantitatively strikingly different for the various species in question. The notable differences found in the fatty acid patterns of the various red cell membranes are remarkable, since the general function of the red cells in mammals will be the same, so these differences prompt the question whether there is any relationship between the specific lipid composition and the permeability properties, which are known to vary widely.

Among the frequent literature references on the possible relations between chemical composition of red cell surfaces and their permeabilities, it was HÖBER[10] who assumed on the grounds of the data available in 1935 that the area of the cell surfaces occupied by lipid, must differ from species to species, to explain their differences in permeability to lipid soluble compounds. PARPART AND DZIEMIAN[11] suggested in 1940 that the permeability should be dependent on the ratio of different types of lipids and on the amount of lipoprotein complexes. In a more recent paper PARPART AND BALLENTINE[4] remarked that no major differences in the proportions of lipid types have been found in the red cells of various species, nor has any significant correlation between lipid content and the large permeability differences between the red cells of various vertebrates been observed. A comparison of our gas-chromatographic data on the fatty acid composition with figures on the permeability properties of the membranes as given in the literature, discloses that such a relationship might exist, though being of another kind than was assumed by these authors.

In Fig. 2 are shown in the first place for each animal the means of the values for

the different fatty acids of Table I. From rat *via* man, rabbit, pig, horse and ox to sheep a steady decrease is shown in the amount of palmitic acid, accompanied by an increasing amount of oleic acid in the ghost membrane lipids. In the second place in Fig. 2 pictures are shown on the permeability of the membranes of the same animals as given by JACOBS, GLASSMAN AND PARPART[12], and by HÖBER AND ØRSKOV[13]. They measured the hemolysis times of red cells in isotonic solutions and figured out that there may be a relation between permeability (P) and hemolysis time (T), as given by $P = c/T$, in which c is a constant[12]. In the pictures the hemolysis times in an isotonic solution of urea, thiourea, glycerol and ethylene glycol respectively are plotted on four perpendicular axes in a logarithmic scale. Rather surprising was the fact that by virtue of these permeability pictures, the studied animals could be placed in the same sequence as on the grounds of the fatty acid analyses, showing this time a gradual shift in permeability properties.

Perhaps no less important is the steady increase in quite the same order in the total percentage of unsaturated acids in the membranes, as can also be seen from Fig. 2 (heavy line). This appears to justify the assumption made by BOOIJ AND BUNGENBERG DE JONG[2], that one of the factors influencing the permeability, should be "the number of double bonds in the carbon chain of the lipids".

In Fig. 2 the hemolysis picture of the horse had to be omitted, as the consulted literature did not provide complete data on this animal. In Fig. 3 the possible relation is demonstrated between the permeability to glycerol and the concentration of oleic acid, one of the most conspicuous acids*. Here can be seen that also the hemolysis time of horse erythrocytes in an isotonic solution of glycerol, given by JACOBS *et al.*[14] in an older publication, is in accord with the parallel found between permeability and fatty acid composition of the lipid moiety of the studied membranes.

The facts appear to support, so far, the assumption that the lipid part of the various biological interfaces may contribute to their distinction and specificity. The noticed differences in the fatty acid pattern of the studied homologue membranes need additional research in several directions to fully justify a general conclusion. One problem, which would perhaps at the moment be difficult to dissolve, concerns the possible differences in fatty acids of the various interfaces of different tissues within one species. Within the scope of the specificity problem of the red cell membrane, not only an extension to other classes of vertebrates is desirable, but it appears also valuable to approach the problem indirectly by determining the influence of diet components on the specific fatty acid composition of the membranes. Whereas an augmentation of the proportion of unsaturated fatty acids in the diet is known to alter the component fatty acids of depot fats remarkably, we suppose that such a reflection in those membrane fatty acids having specific functions, must occur to a less extent. Furthermore, it seems interesting to make a study of the red cell membranes of organisms under pathological conditions.

Another way of tackling the problem in discussion may be a determination of the fatty acids of the different types of lipids in the various red cell membranes. At our laboratory work is in progress now to differentiate between fatty acids from

* The correlation coefficients (r) have been determined between the hemolysis times for glycerol (a) and glycol (b) and the amounts of palmitate (x) and oleate (y) in the ghost lipids, and were found to be significant.

$r_{ax} = -0.905$, $p < 0.01$; $r_{ay} = +0.773$, $p < 0.05$; $r_{bx} = -0.956$, $p < 0.001$; $r_{by} = +0.868$, $p = 0.01$

neutral lipids, lecithins and other phosphatides by analysing these fractions of ghost membrane lipids one by one.

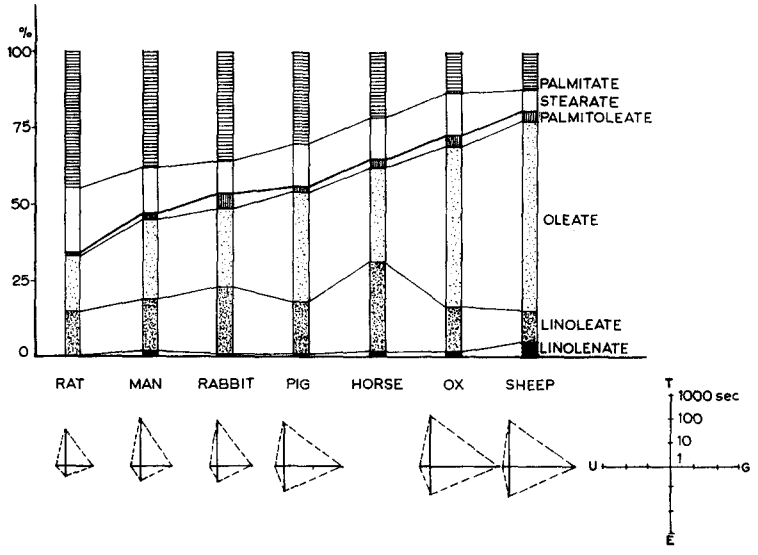

Fig. 2. Comparison of fatty acid percentages of ghost lipids with permeability properties of red cells. Upper part: Means of the fatty acid methyl ester values from Table I, omitting the minor components laurate and myristate. The heavy line separates saturated and unsaturated acids. Lower part: G, hemolysis times in isotonic solutions of glycerol; E, ethylene glycol; U, ureum; and T, thioureum, respectively, on a logarithmic scale as indicated. The picture for the pig is calculated from values of HÖBER *et al.*[13], the other pictures are taken directly from JACOBS *et al.*[12]. No complete values for the horse are given by these authors.

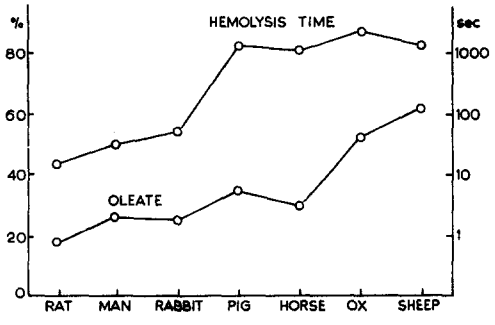

Fig. 3. Comparison of oleic acid content of the ghost lipids with the permeability for glycerol. Lower curve: Mean percentages of oleate from Table I (left hand ordinate). Upper curve: Hemolysis times of erythrocytes in isotonic solution of glycerol[12–14] (right hand ordinate).

We are aware that the results obtained so far, need extensive supplementary evidence to secure more general conclusions on the relation between fatty acid composition and permeability properties of membranes, conclusions which may be fruitful for a further elucidation of structure and mechanism of biological interfaces.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. W. A. J. BORG for his interest during the work and for critically reading the manuscript, and to Dr. K. PUNT for kindly supplying some samples of human blood.

REFERENCES

1 K. C. WINKLER, *Thesis*, Leiden, 1938.
2 H. L. BOOIJ AND H. G. BUNGENBERG DE JONG, *Protoplasmatologia*, Vol. I, 2, Springer, Vienna 1956, p. 141.
3 W. D. STEIN AND J. F. DANIELLI, *Discussions Faraday Soc.*, 21 (1956) 238.
4 A. K. PARPART AND R. BALLENTINE, in E. S. G. BARRON, *Modern Trends in Physiology and Biochemistry*, Acad. Press, New York 1952, p. 135.
5 A. K. PARPART, *J. Cellular Comp. Physiol.*, 19 (1942) 248.
6 F. R. CROPPER AND A. HEYWOOD, *Nature*, 174 (1954) 1063.
7 A. T. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 679.
8 S. R. LIPSKY, R. A. LANDOWNE AND M. R. GODET, *Biochim. Biophys. Acta*, 31 (1959) 336.
9 H. WAGNER, L. ABISCH AND K. BERNHARD, *Helv. Chim. Acta*, 38 (1955) 1536.
10 R. HÖBER, *J. Cellular Comp. Physiol.*, 7 (1936) 367.
11 A. K. PARPART AND A. J. DZIEMIAN, *Cold Spring Harbor Symposia Quant. Biol.*, 8 (1940) 17.
12 M. H. JACOBS, H. N. GLASSMAN AND A. K. PARPART, *J. Exptl. Zool.*, 113 (1950) 277.
13 R. HÖBER AND S. L. ØRSKOV, *Arch. ges. Physiol., Pflüger's*, 231 (1933) 599.
14 M. H. JACOBS, H. N. GLASSMAN AND A. K. PARPART, *J. Cellular Comp. Physiol.*, 7 (1935) 197.

Biochim. Biophys. Acta, 43 (1960) 95–103

QUELQUES REMARQUES COMPLÉMENTAIRES SUR L'HYDROLYSE DES TRIGLYCERIDES PAR LA LIPASE PANCRÉATIQUE

M. J. CONSTANTIN, L. PASERO ET P. DESNUELLE

Laboratoire de Chimie Biologique, Faculté des Sciences, Marseille (France)

(Reçu le 18 février 1960)

SUMMARY

Some additional remarks on the hydrolysis of triglycerides by pancreas lipase

1. *In vitro* lipolysis of triglycerides is impeded by difficulties due to the conflict between hydrolysis, on the one hand, and progressive inactivation of the lipase, on the other. The maximum rate of hydrolysis attainable consequently depends in the first place on the relation between the amounts of enzyme and substrate present. It is found that, when the initial proportion of the enzyme is increased, the lipolysis tends to become total.

2. However, even when lipolysis is ultimately total, considerable quantities of transitory partial glycerides are formed while lipolysis is going on.

3. The "activating" effect on lipolysis commonly attributed to calcium ions and bile salts is discussed.

4. Some consequences of these *in vitro* observations on the nature of the products absorbed by intestinal mucosa and on the mechanism of triglyceride resynthesis in this mucosa, are briefly considered.