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UNSATURATED FAT OXIDATION IN BONE MARROW HOMOGENATES

By

Marian W. Kies and Ann Webster

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CARBIDE AND CARBON CHEMICALS CORPORATION
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ABSTRACT

UNSATURATED FAT OXIDATION IN BONE MARROW HOMOGENATES

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Because of the high fat content of bone marrow and the fact that the tissue is so important metabolically, a study was undertaken of the oxidation of certain fatty acids and their derivatives in bone marrow. The unsaturated long chain fatty acids were investigated because less is known of their physiological fate than that of the saturated acids, although the susceptibility of the unsaturated fats to oxidation in the presence of heme derivatives has been suggested as a basis of their function. Also of interest was the possible existence in animal tissue of an enzyme similar to the plant enzyme, lipoxidase.

Linoleic acid and related compounds have been found to increase the oxygen uptake of bone marrow homogenates. A component of bone marrow fat also increases oxygen consumption of marrow preparations. Since a compound similar to linoleic acid is known to be present in bone marrow fat, it is suggested that the oxidation of this compound may play a role in bone marrow metabolism.

Linoleic acid oxidation by bone marrow preparations is probably not enzymatic because the active principle is heat stable. In view of the erythropoietic function of bone marrow, a hemin-type catalysis is suggested. Like many heme proteins responsible for other types of catalysis in animal tissue, this catalyst is inhibited by cyanide. Such inhibition is not considered proof that the substance is a heme derivative.

The fact that the reaction is non-enzymatic does not detract from its possible importance in bone marrow metabolism. Coupled oxidation reactions in lipoxidase and fat autoxidation systems are well-known and may also occur in bone marrow. Fat peroxide, which accumulates in homogenized marrow under certain conditions, could serve as the peroxide donor in a peroxidase system and might be of some importance in this respect.

Preliminary fractionation experiments indicate that the oxidative catalyst is water-soluble and can easily be separated from the hemoglobin in bone marrow extracts by saturation with potassium dihydrogen phosphate.

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UNSATURATED FAT OXIDATION IN BONE MARROW HOMOGENATES

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Considerable work has been done on the fat content of bone marrow and its physiological and pathological variations, but very little attention has been paid to the possible significance of fat in bone marrow metabolism. Krause (1) has studied the effect of anemia on the fat content of marrow. Barron and co-workers (2) in a recent article stated that fatty acids increased the *in vitro* respiration of bone marrow but gave no details as to the compounds tested. Other studies of bone marrow respiration have been concerned more with carbohydrate than with fat metabolism (3, 4). Because of the high fat content of this tissue, figures in the literature (1, 5, 6) vary from 20 to 80 per cent or more depending on the origin of the bone marrow, the condition of the animal, the method of extraction, etc.-and because of the fact that it is so important metabolically, a study was undertaken of the oxidation of certain fatty acids and their derivatives in bone marrow.

The unsaturated long chain fatty acids were investigated since less is known of their physiological function than that of the saturated acids (cf. Lehninger's work) (7). Although very little is known of the place in animal metabolism occupied by the nutritionally essential fatty acids (linoleic, linolenic and arachidonic) their susceptibility to oxidation in the presence of hemin and heme derivatives has been suggested as a basis of their function (8). Also of interest is the possible existence in animal tissue of an enzyme similar to the plant enzyme, lipoxidase, which

has been shown by Balls, Axelrod and Kies (9) to oxidize specifically only those unsaturated acids which contain a series of methylene interrupted double bonds. This observation was confirmed by Burr and Holman (10). Hove (11) has published data showing the presence of a lipoxidase-type enzyme in gastric mucosa.

EXPERIMENTAL

The oxygen uptake of bone marrow is not great compared to that of other animal tissues, but it is nevertheless of sufficient magnitude to study in the conventional Warburg apparatus. Mixed fresh marrow obtained from the long bones of normal rabbits was used in the experiments, as well as marrow which had been frozen and stored at -36° C. Some of the frozen marrow was lyophilized and extracted with petroleum ether for further study.

The animals were killed by a blow on the head, the long bones removed, the ends of the bones cut off with bone cutters, and the marrow blown out of the bones with compressed air (this means of obtaining the marrow without much damage to the structure was suggested by Huggins (6) and considerably shortened the time spent in collecting the tissue.)

Characteristics of Bone Marrow Fat: Since the fat content of bone marrow has been the subject of several publications (1, 5, 6) no attempt was made to study this extensively. For purposes of comparison however, several preparations were weighed before lyophilization and the weight of fat obtained from the various samples was recorded. The values are given in Table I.

Table I
Fat Content of Various Rabbit Marrow Preparations

Wt. of Fresh Marrow	Wt. of Fat	Iodine Value
gm.	gm.	
1.5	0.4	---
5.8	1.8	135
4.5	1.6	117
7.4	3.1	116
3.0	1.9	---
2.3	0.9	---

Petroleum ether[1/] was used as the solvent. The tissue was lyophilized, coarsely ground and left standing under petroleum ether overnight. The residue was then homogenized with fresh solvent and filtered. Traces of solvent were removed *in vacuo* and the dry tissue stored at 5° C. The ether was removed from the fat by evaporation at room temperature, the last traces being removed by evacuation as before. The fat was then stored at -36° C.

The iodine values given in Table I were determined with the Rosenmund-Kuhnhenn reagent which consists of pyridine sulfate dibromide (13).

One sample of bone marrow fat was obtained by stirring an ice-cold homogenate for an hour or more until the fat formed a cake on top of the liquid and around the stirrer. The fatty layer was washed with water and suspended in acetone. Only a very small portion dissolved and this acetone-soluble fraction had an iodine value of 129. The presence of fatty acids more unsaturated than oleic acid is indicated by the iodine values found for the different samples of bone marrow fat. This was verified by heating the sample with KOH in glycerol to isomerize any linoleic, linolenic or arachidonic acids present (14). The ultra-violet absorption of 50 mg. bone marrow fat treated in this manner was almost identical with that of 10 mg. isomerized linoleic acid (Fig. 1). The presence of linoleic acid in ox bone marrow fat was observed by Hilditch (15) who cited the similarity of bone marrow fat to perinephric and rump fats. Cheng's (16) data also indicated the presence of a fatty acid more unsaturated than oleic, but he did not identify this fraction.

Bone Marrow Respiration--Effect of Fats and Fatty Acids on Oxygen Uptake: Most of the respiratory studies were done with bone marrow homogenized in 10 volumes of water with a glass homogenizer. Homogenization in dilute phosphate buffer did not alter the rate of oxygen uptake. All

1/ Purified by treating with alumina to remove the peroxides. (12)

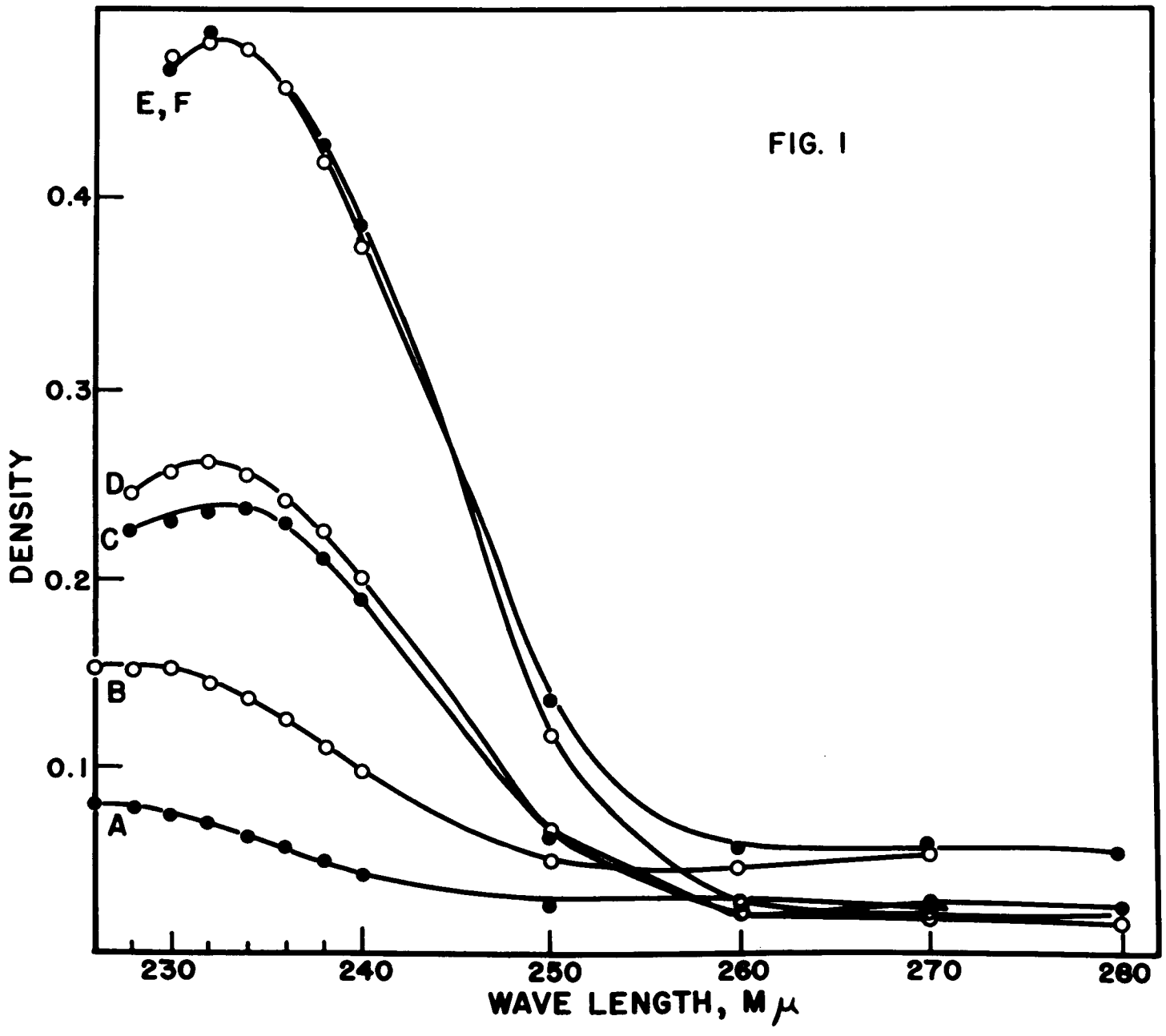

E, F
D
C
B
A
0.4
0.3
0.2
0.1
DENSITY
230
240
250
260
270
280
WAVE LENGTH, Mμ

FIG. 1

7a

respiration studies were carried out at 37° C. with air as the gas phase. Values given in the tables are averages of duplicate determinations, unless otherwise stated. If the duplicates did not agree within 10 per cent, both values are given. The total volume in each flask was 4 ml. and the amounts of tissue and reagent used in this volume are specified in the footnotes under each table and figure. The phosphate buffer and acetone concentrationsare final concentrations after dilution to 4 ml.

There was considerable variation in the oxygen uptake of marrows obtained from different animals. Attempts to show that this variation was due to the mode of handling the samples were unsuccessful. It was finally concluded that the variation was inherent in the animals themselves (see Warren (17)). Excellent agreement among aliquots of a single preparation was obtained, and conclusions were drawn therefore from comparative results obtained with the same tissue preparation.

The effect of added linoleic acid on oxygen uptake of bone marrow homogenates was marked and consistent. Regardless of the extent of residual respiration, addition of linoleic acid either in acetone solution or in dilute alkali, increased the oxygen uptake several hundred per cent. (See Tables II - VIII, and Fig. 2). The effect of linoleic acid was more striking when lyophilized, defatted marrow was used because the residual respiration was small. Bone marrow fat was also capable of stimulating oxygen uptake of the fat-free residue and, under certain conditions, the respiration of fresh marrow fractions (Table II). The susceptibility of bone marrow fat to oxidation by various bone marrow preparations was not as great as that of linoleic acid. This may be attributed to various factors--the low concentration of unsaturated substrate in bone marrow fat; the greater solubility of the free acid, the possibility of fat-soluble inhibitors in the bone marrow fat samples, etc.

Table II

Oxidation of Bone Marrow Fat

	Oxygen Uptake
	cmm. per hour
10 mg. bone marrow residue 1/	6
Ditto, plus 4 mg. linoleic acid	39
Ditto, plus 26 mg. bone marrow fat 2/	48
10 mg. bone marrow residue plus 23 mg. bone marrow fat 2/	34,42
0.25 mg. hemin from rabbit blood plus 23 mg. bone marrow fat	38,48
23 mg. bone marrow fat alone	3
10 mg. bone marrow residue plus 20 mg. linoleic acid	97,78 (in 10 min.)
0.25 mg. hemin plus 20 mg. linoleic acid 3/	92 (" " "
1 ml. 1-10 extract of homogenized marrow	7
Ditto, plus bone marrow fat 4/	77,41
Equiv. insol. material inactive	
200 mg. bone marrow homogenized in ice water so that fatty layer separated (10 per cent acetone in flasks)	16
Ditto, plus equiv. amt. of separated fat dissolved in acetone	42

1/ Lyophilized, defatted residue suspended in 4 ml. 0.025 M phosphate, pH 7.6 (10 per cent acetone).

2/ Fat extracted from lyophilized marrow with petroleum ether. After the solvent was evaporated, the fat was suspended in acetone and filtered.

3/ 10 per cent acetone in the bone marrow flasks. Linoleic acid dissolved in dilute sodium hydroxide. 0.075 M phosphate pH 6.7, in all flasks.

4/ Ice-cold homogenate stirred until fatty layer separated. The fat was suspended in acetone and filtered. 40 per cent of the acetone-soluble fat originally present in 1 ml. homogenate was used.

Table VIII

Bone Marrow Fractionation

	Oxygen Uptake (cmm. per hour)	
	No substrate	4 mg. linoleic acid
A. 1 ml. 1-10 homogenate of bone marrow 1/	0	73
B. 1 ml. extract of A	0	67,77
(B. was saturated with KH_2PO_4, left 24 hrs. at 5° C.,2/ and centrifuged. The supernatant retained all of the original color. The small amount of gray precipitate was redissolved in dilute NaOH (pH 10-11), the solution (C) neutralized to pH 7.5 and diluted to volume of original extract.		
C. 1 ml. solution	0,4	55
(Solution C. was saturated with KH_2PO_4 and ppt. redissolved as before (C-2).		4/
C-2 1 ml. solution 3/	10,14	70
1 ml. solution heated, 90-100., 1 min.		71
1 ml. solution in 0.02 M NaCn 5/		33,38

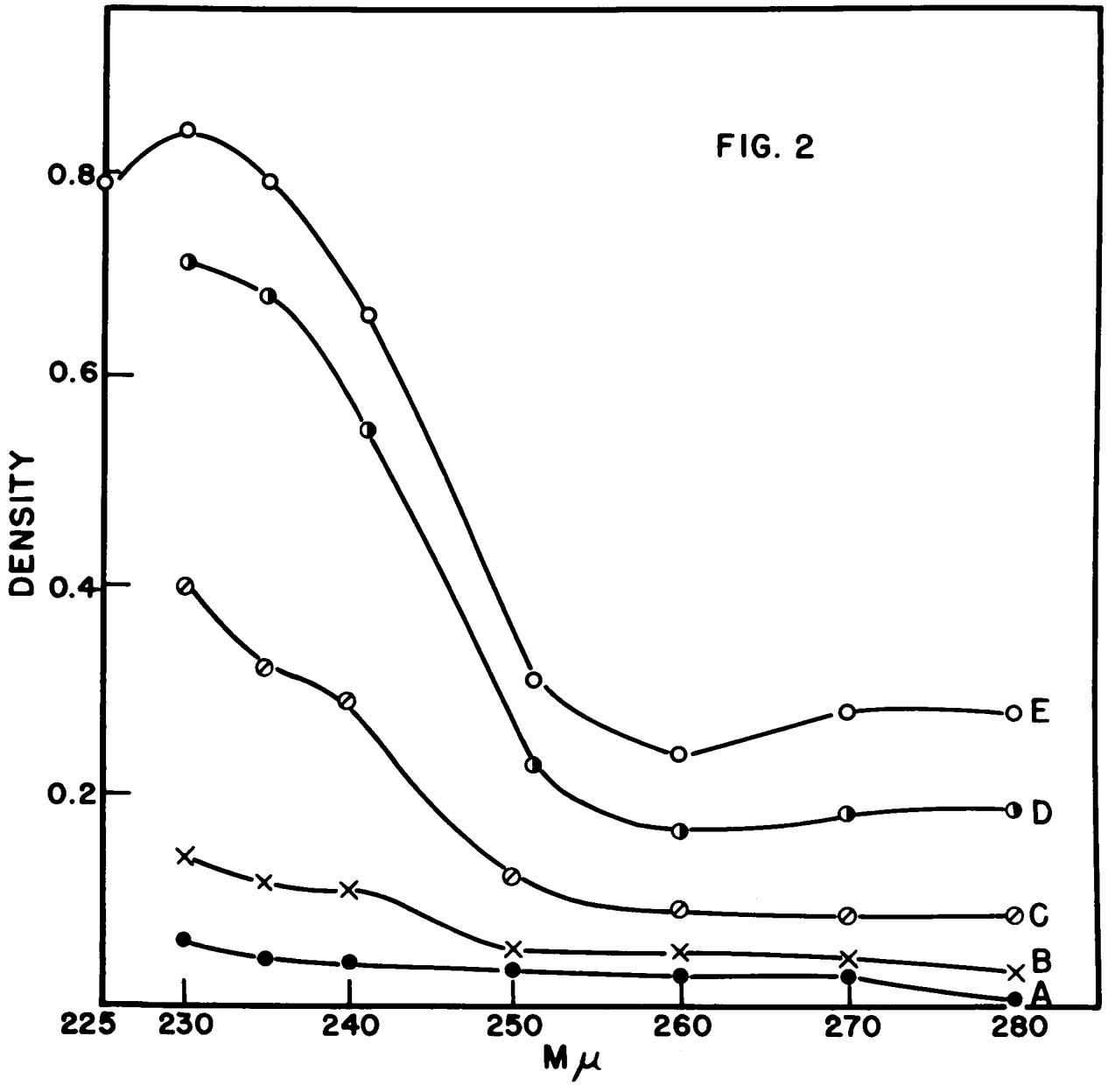
1/ In 4 ml. 0.025 M KH_2PO_4 (10 per cent acetone). Same conditions throughout.

2/ Saturation with NaCl did not cause any precipitation; 0.5 saturated ammonium sulfate precipitate was about 3/4 as active as the KH_2PO_4 precipitate; 50 per cent acetone precipitated about half the activity but also brought down all the color in the original extract.

3/ 1 ml. of this solution contained just enough protein to give a turbidity when diluted with 10 ml. 2.5 per cent tricholoracetic acid; 1.5 ml. did not give a positive test for iron by the Kennedy procedure (25).

4/ Oxygen uptake determined with alkali in the center well. R. Q. determined by the "direct method" was 0.2 for this reaction.

5/ No alkali in center wells. Cyanide solution was freshly prepared and neutralized before use.



11a

Other unsaturated fatty acids and esters also enhanced the oxygen uptake of bone marrow homogenates *in vitro* (Table III). As was expected from previous work on the catalytic specificity of hemin and lipoxidase (8, 9) the esters of linoleic and linolenic acids behaved like linoleic acid. The results with oleic acid were variable. It is ordinarily not as active as the more unsaturated acids in oxidation experiments and the stimulation of bone marrow respiration by oleic acid shown in Expt. 2 in the table was a little surprising. This marrow was darker colored than the usual preparations and showed a higher residual oxygen uptake. Later experiments with other marrows did not show any appreciable oxidation of oleic acid.

It should be noted that it was necessary to include acetone in experiments on bone marrow fat and the unsaturated fatty esters in order to obtain adequate emulsions. Early experiments indicated that acetone alone enhanced the oxygen uptake of bone marrow although this increase was never as great as the effect of the unsaturated compound tested. It was assumed that this effect of acetone might be due to its influence on the emulsification of the bone marrow fat present in the sample. However, the influence of acetone was variable, ranging all the way from a marked increase in oxygen uptake to a definite depression. In all experiments which required the use of acetone as a fat solvent, equivalent volumes of acetone were added to the controls.

Effect of Hydrogen Ion Concentration on Bone Marrow Respiration: Several of the experiments recorded in the present study were carried out in the presence of acid phosphate because it was found that the oxygen uptake of bone marrow was considerably enhanced by the addition of either acid phosphate or dilute hydrochloric acid (Fig. 3). The experiment summarized in Fig. 3 did not include a comparison of phosphate and HCl as the acidifying agents. Data from other experiments proved that the stimulation was a pH effect independent of the presence of phosphate.

Table III

Oxidation of Pure Unsaturated Fatty Acids and Esters by Bone Marrow Preparations

Substrate	Oxygen Uptake (cmm. per hour)
Control 1/	0
3 mg. methyl oleate	5
3 mg. methyl linoleate	29
3 mg. methyl linolenate	44
3 mg. oleic acid	20
3 mg. linoleic acid	32
Control 2/	40
4 mg. oleic acid	205
4 mg. linoleic acid	300
4 mg. methyl linoleate	192
4 mg. methyl linolenate	156,227
Control 3/	9
4 mg. oleic acid	17
4 mg. linoleic acid	48
Control 4/	3,12
4 mg. oleic acid	0, 8
4 mg. linoleic acid	42 (single value)

1/ 100 mg freshly homogenized marrow, suspended in 4 ml. 0.025 M phosphate, pH 7.3 (10 per cent acetone).

2/ 200 mg. freshly homogenized marrow, suspended in 0.025 M KH_2PO_4 (10 per cent acetone), final pH 6.0-6.2.

3/ 100 mg. freshly homogenized marrow, suspended in 0.025 M KH_2PO_4. Linoleic and oleic acids dissolved in dilute sodium hydroxide before adding to the buffered homogenate. Final pH 6.0-6.2.

4/ 100 mg. homogenized marrow suspended in 0.025 M KH_2PO_4 (10 per cent acetone). This was the same marrow as 3/, frozen 24 hours before the experiment.

The compounds tested were obtained from Hormel Foundation. All showed no autoxidation in one hour under the conditions tested.

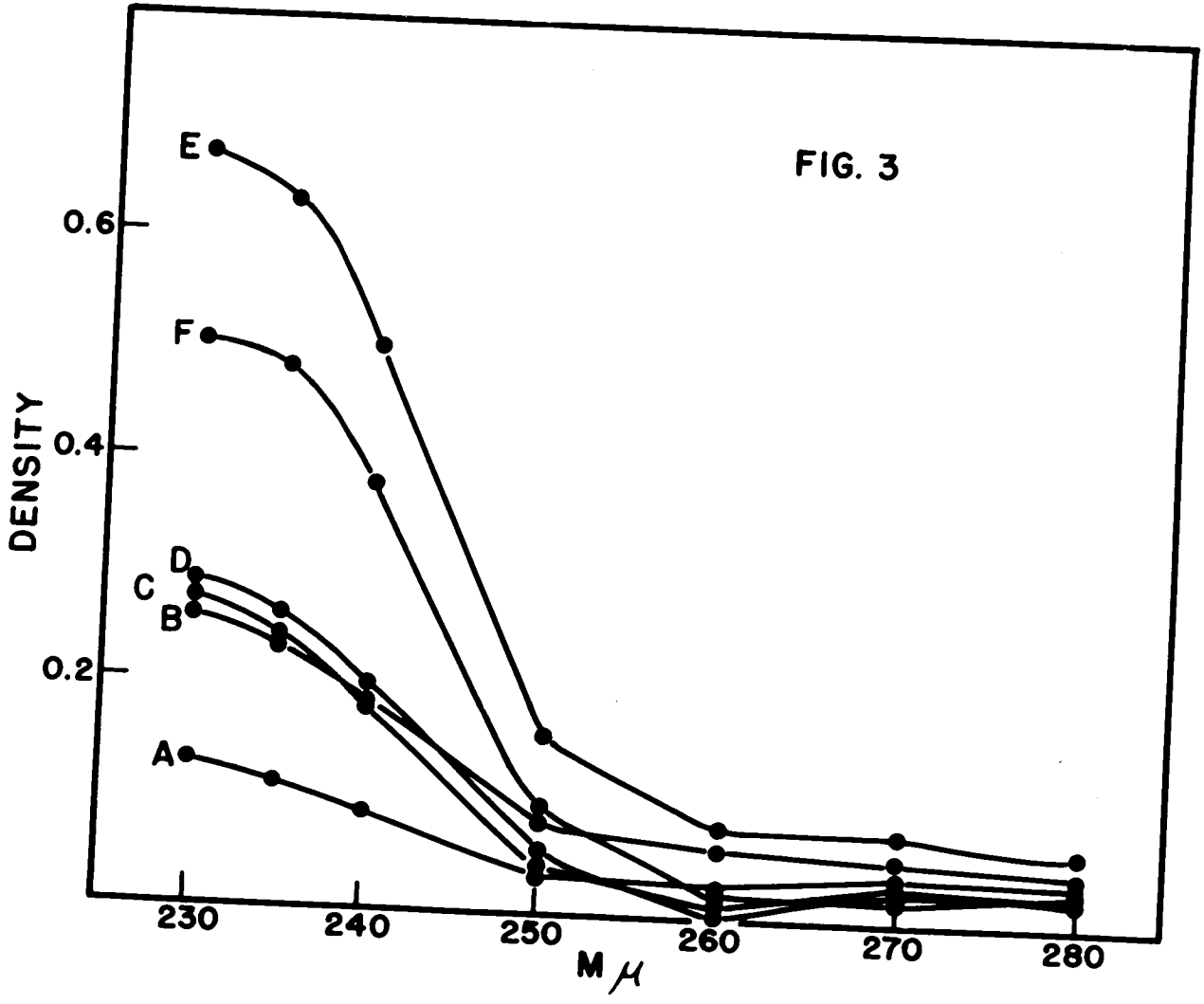


FIG. 3

14a

Effect of Reagents Other Than Fats on Bone Marrow Respiration: The homogenates used in this series of experiments had a lower Q-O_2 than other workers have gound for bone marrow but this was not unexpected, because most of the experiments reported previously have been carried out on tissue slices or cell cespersions. Because of the well-known effect of cytochrome c and adenosine triphosphate on respiration of tissue homogenates, the two compounds were added to homogenized bone marrow to test their effect on its oxygen uptake. 0.5 mg. cytochrome c per 100 mg. freshly homogenized bone marrow had no influence on the rate of oxygen uptake. The stimulating effect of adenosine triphosphate is shown in Fig. 4 Barron (2) states that they found only a slight enhancement of bone marrow respiration with this compound. In view of the variability of the residual respiration of different marrow preparations, it is probable there are also variations in the concentrations of various physiological constituents of the tissue, and this might account for the discrepancy between these results and Barron's.

Another compound known to have a marked stimulating effect on the oxidation of lipids by various tissues is ascorbic acid (19, 20). No evidence of a similar type of catalytic oxidation in bone marrow homogenates, with or without added linoleic acid, was found (Table IV).

Increased bone marrow respiration in the presence of serum is well established. Warren (17) has shown that the active principle is present in the ultrafiltrate of serum and is organic in nature. Although this phenomenon was not observed consistently in our experiments, there was one noticeable effect of serum, as shown in Table IV, that is, inhibition of linoleic acid oxidation. This may be caused by combination between linoleic acid and the serum proteins or (in Expt. 3, at least) it could be caused by complexing of the substrate with traces of hemoglobin present.

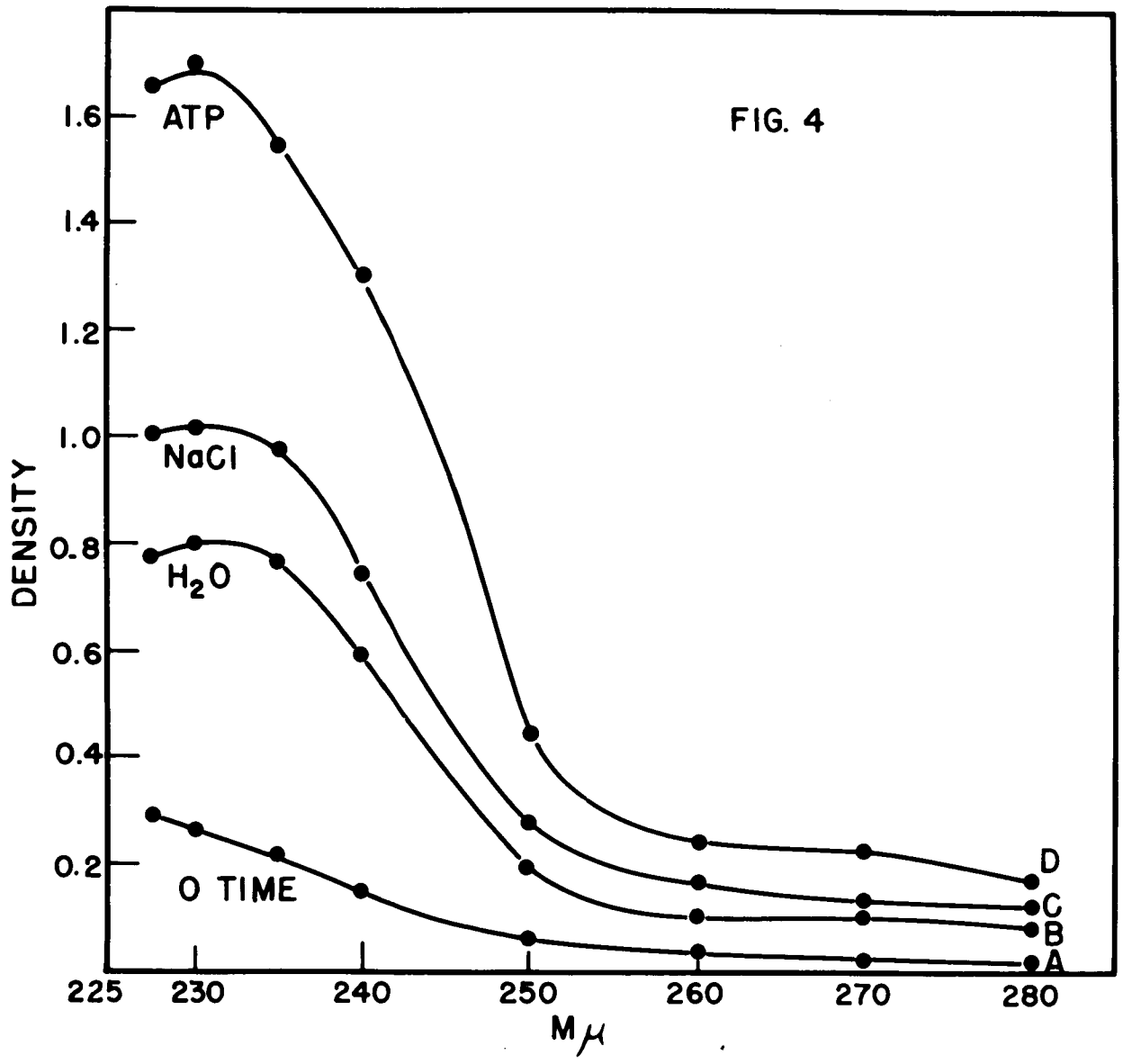


FIG. 4
ATP
NaCl
H_2O
O TIME
DENSITY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
225
230
240
250
260
270
280
Mμ
D
C
B
A

16a

Table IV

Effect of Ascorbic Acid and Serum on Bone Marrow Respiration and Linoleic Acid Oxidation

	Cmm. Oxygen Uptake 1 hr.	2 hr.
Bone marrow alone 1/	19	
Ascorbic acid alone	34	
Linoleic acid alone	5	
Bone marrow plus ascorbic acid	22,27	
Bone marrow plus linoleic acid	57	
Bone marrow, ascorbic acid and linoleic acid	66	
Ascorbic acid plus linoleic acid	24	
2 ml. 1-10 homogenate of bone marrow in serum 2/	12	27
Ditto, plus 4 mg. linoleic acid	14	31,24
2 ml. 1-10 homogenate of bone marrow in 0.9 per cent NaCl	3	12 (single values)
Ditto, plus 4 mg. linoleic acid	72,32	360,298
2 ml. serum	0	1,6
Ditto, plus 4 mg. linoleic acid	2	3
1 ml. 1-10 dispersion of bone marrow in serum 3/	6	14
Ditto, plus 4 mg. linoleic acid	76	123
1 ml. 1-10 dispersion of bone marrow in water	6	9
Ditto, plus 4 mg. linoleic acid	150	212
1 ml. serum	15,5	8
Ditto, plus 4 mg. linoleic acid	106,85	117

1/ 200 mg. freshly homogenized marrow suspended in 4 ml. 0.025 M phosphate, pH 6.7. 1 mg. ascorbic acid and 4 mg linoleic acid, as specified. Same buffer used in all flasks.

2/ Dilutions were made with physiological saline to prevent hemolysis of any red cells present.

3/ Bone marrow suspended in serum or water and the cells dispersed simply by shaking by hand. The linoleic acid oxidation observed in this serum sample was undoubtedly caused by hemoglobin, since conditions permitted hemolysis of any red cells present.

Accumulation of Fat Peroxide in Bone Marrow Homogenates: Since unsaturated fatty acids and esters enhanced the oxygen uptake of bone marrow homogenates, an attempt was made to isolate the fat fraction after incubation to determine whether the extra oxygen had gone into fat peroxide formation. This was also done in experiments which did not involve the addition of substrate. Several methods of fat extraction were tried, but none were entirely satisfactory. Any quantitative correlation between oxygen uptake and peroxide formation in bone marrow would require more intensive study of the problem of fat extraction. As shown in Figs. 2, 3, and 4 the ultraviolet absorption spectra of the extracts indicate increased peroxide formation with increased oxygen uptake, but the figures cannot be interpreted quantitatively.

Heat Stability of Oxidative Catalyst in Bone Marrow: The substance in bone marrow responsible for unsaturated fat oxidation is quite different from the well-known plant lipoxidases in its stability to heat (Table V). In this respect it is similar to a fat oxidative catalyst isolated from liver by Libet & Elliott (19).

Cyanide Inhibition of Linoleic Acid Oxidation: Although cyanide is generally considered to be a specific inhibitor for enzymatic or catalytic systems depending on hemin derivatives for their activity, it has been shown (21, 22, 23) that hemin-catalyzed unsaturated fat oxidation is not inhibited by cyanide. It is interesting to note, however, that in the present series of experiments the increased respiration caused by addition of linoleic acid to bone marrow homogenates is completely inhibited by cyanide whereas the residual respiration is only partially inhibited (Table VI).

Table V

Heat Stability of Oxidative Catalyst in Bone Marrow

	Oxygen Uptake (cmm. per hour)	
	Unheated	Heated 1/
100 mg. frozen bone marrow, homogenized suspended in 4 ml. 0.025 M phosphate, pH 7.6 (10 per cent acetone)	27, 39	47,68
Ditto, plus 4 mg. linoleic acid	130,162	133
2 ml. 1-10 extract of sliced bone marrow 2/ plus 4 mg. linoleic acid	62,75	69,80

1/ Sample was heated with stirring in a water bath. About 4 min. were required to raise the temperature to 90° C., and the sample was left in the bath 1 min. after the water started to boil. Re-homogenized after heating.

2/ Extraction more complete than usual, approximating the results obtained when bone marrow was homogenized before extraction.

Table VI

Effect of Cyanide on Fatty Acid Oxidation in Bone Marrow

	Cmm. Oxygen Uptake per Hour	
	-----	0.02 M NaCN 1/
200 mg. freshly homogenized bone marrow suspended in 4 ml.	32,47	20
Ditto, plus 4 mg. linoleic acid	119	35
10 mg. bone marrow residue suspended in 4 ml. 2/ 0.025 M phosphate, pH 7.6 (10 per cent acetone)	6	7
Ditto, plus 4 mg. linoleic acid	39	6
Ditto, plus 26 mg. bone marrow fat 2/	48	19

1/ Final concentration. Cyanide solutions were freshly prepared and neutralized before use. The use of cyanide in the center well to prevent diffusion of HCN from the main space, as described by Robbie (24), made no difference in the extent of cyanide inhibition.

2/ See Table II.

Effect of Aging and Dialysis on Respiratory Activity of Bone Marrow Homogenates: Much has been said about the importance of using freshly prepared homogenates in studies of tissue respiration. The variability observed in bone marrow respiration during the course of this work led to some experiments on the effect of aging on oxygen uptake. The results were entirely unexpected--instead of causing a decrease in the respiration of the homogenate, storage at 5° C. for 24 hours caused a marked increase (Table VII).

Table VII

Effect of Aging and Dialysis on Respiratory Activity of Bone Marrow Homogenates

	Oxygen Uptake (cmm. per hour)	
	No substrate	4 mg. linoleic acid
200 mg. homogenized bone marrow 1/	25	99,77
Same homogenate after 24 hrs. at 5° C.	88	158,121
Same homogenate after dialysis against distilled water, 24 hrs., 5° C.	143	121
Same homogenate after 72 hrs. Dialysis	168,137	112

1/ Suspended in 4 ml. 0.025 M KH_2PO_4 (10 per cent acetone) for experiment. No phosphate added during aging and dialysis.

Dialysis, which is known to remove substrates available for ordinary tissue respiration, only served to increase further the oxygen uptake of the aged sample. After dialysis addition of linoleic acid no longer caused an increase in the oxygen uptake of the homogenate, but actually depressed the augmented respiration observed in the dialyzed homogenate.

Fractionation of Bone Marrow: The catalyst responsible for the oxidation of linoleic acid in bone marrow was found to be associated with solid material when bone marrow slices were extracted. When the tissue was homogenized before extraction, presumably destroying the cell structure, a large part of the activity was found in the extract. That this activity was

not associated with the hemoglobin present in the extract was shown by the fractionation procedure outlined in Table VIII.

SUMMARY AND CONCLUSIONS

Linoleic acid and related fatty acids and esters have been found to increase the oxygen uptake of bone marrow homogenates. The petroleum ether-soluble fraction of lyophilized marrow also increases oxygen consumption of marrow preparations. Since a compound similar to linoleic acid is known to be present in bone marrow fat, it is suggested that the oxidation of this compound may play a role in bone marrow metabolism.

In the present study no attempt has been made to differentiate the various fractions of bone marrow fat. The bone marrow fat referred to in Table II consisted of the acetone-soluble fraction of the total lipids.

The oxidation of linoleic acid by bone marrow preparations is probably not enzymatic in nature because the active principle is heat stable. In view of theerythropoietic function of bone marrow, a hemin-type catalysis is suggested. Like many heme proteins responsible for other types of catalytic reactions in animal tissue, the catalyst is inhibited by cyanide. Such inhibition is not considered proof that the substance is a heme derivative.

The fact that the reaction is non-enzymatic does not detract from its possible importance in bone marrow metabolism. Coupled oxidation reactions in lipoxidase and fat autoxidation systems are well-known and may also occur in bone marrow. Fat peroxide, which accumulates in homogenized marrow under certain conditions, could serve as a peroxide donor in a peroxidase system and might be of some importance in this respect.

Preliminary fractionation experiments indicate that the activity is distinct from the hemoglobin present in bone marrow extracts.

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