Summary. The K_m for brain 5HTPD is 5.2×10^{-5} M. The inhibition of this enzyme by phenylalanine derivatives is competitive and substrate-dependent at pH 8.05. This inhibition does not appear to play a major role in the decrease of brain 5HT reported in experimental phenylketonuria.

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Relative Rates of Oxidation of Palmitate-1-C^{14} by White Blood Cells, Red Cells and Platelets of Rats.* (29834)

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Whole blood cells have been reported to incorporate acetate-C^{14} into lipids(1,2), to oxidize palmitate-1-C^{14} to $C^{14}O_2$(3) and to incorporate fatty acids into phosphatides, triglycerides and esters of cholesterol(4). Marks *et al*(5) have found that the rate of acetate-1-C^{14} incorporation into mixed lipids of normal white blood cells, on a per cell basis, averaged 80 times greater than that into the platelet lipids and at least 1000-fold the incorporation into lipids of red cells. The present investigation was done in order to provide comparable data on fatty acid oxidation. A preliminary report has appeared(6).

Methods. The blood of normal, fasting male rats, weighing between 300 and 400 g and killed by decapitation, was collected into an exact volume of isotonic medium (NaCl—0.137 M; KCl—0.004 M; Na_2HPO_4 buffered to pH 7.4—0.01 M), containing heparin (0.04 mg per ml) and then adjusted with the same medium in order to obtain exactly a 7.5 times diluted blood. About 50 ml of this diluted blood were centrifuged immediately at $1800 \times g$ for 30 minutes, to prepare cell-free plasma of the same dilution used to adjust the volumes of various cell populations separated from another aliquot part of the same diluted blood. Samples were also taken from this diluted blood for counts of white cells, platelets and red cells. Siliconized glassware was used throughout.

Separation of white blood cells, platelets and red cells. Various methods of separation of white blood cells, using fibrinogen(7), dextran(8), or other macromolecular substances such as polyvinylpyrrolidone(9) were tested. Many of these methods do not separate platelets, and very soon it was observed that dextran, fibrinogen, polyvinylpyrrolidone and other hydrophilic macromolecular substances, whether proteins (ovalbumin, beta globulin) or not (methyl cellulose, Elvanol polyvinyl

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alcohol) modify the rate of $C^{14}O_2$ evolution from palmitate-1-C^{14} by blood cells when added to the medium of incubation(10). Subsequently the procedure of Shinowara(11) was adopted, which attains the separation of white cells, platelets and red cells by differential centrifugation of citrated whole blood and results in 3 fractions: (a) blood plasma rich in platelets, separated by centrifugation at 400 $\times$ *g* for 15 minutes, (b) "buffy coat" rich in white cells, obtained by centrifugation at 1650 $\times$ *g* for 30 minutes, (c) red cells pipetted from the bottom of the centrifuge tube, poor in white cells and platelets.

This procedure was modified as follows: an exact volume (example given: 15 ml) of 7.5 times diluted blood was centrifuged at lower speed and for a shorter time than in the original procedure (because the sedimentation of all cellular elements is faster in diluted plasma) and the platelets-rich plasma was removed by pipetting. No second centrifugation was performed, but the majority of red cells were pipetted from the bottom of the centrifuge tube by means of a thin capillary pipette and then resuspended in blood plasma of the same dilution, prepared in advance, and adjusted by the same diluted plasma exactly to the volume of 15 ml. To the white cells of the "buffy coat" remaining in the centrifuge tube after platelets-rich plasma and the majority of red cells were removed, blood plasma of the same dilution was added and the volume adjusted again exactly to 15 ml. Similarly, the volume of platelets-rich plasma was also adjusted to 15 ml by addition of diluted plasma. In this way all the blood cells which were originally present in 15 ml of 7.5 times diluted blood were divided into 3 different cell populations (platelets-rich plasma, white cells of the "buffy coat," the majority of red cells), each suspended in blood plasma of the same dilution and adjusted to the same volume of 15 ml. In each of these 3 cell populations again white cells, platelets and red cells were counted and their sum compared with their counts in the diluted whole blood. Aliquot parts of each of the 3 cell populations in diluted plasma were then incubated for 60 minutes with palmitate-1-C^{14}, and the evolved $C^{14}O_2$ was assayed for radioactivity.

Preparation of the substrate. Palmitic-1-C^{14} acid (specific activity 10.0 millicuries/millimole, from New England Nuclear Corp.) was stored dissolved in benzene. An aliquot of the benzene solution was pipetted into a small volumetric flask and evaporated in a mild stream of air. Palmitic acid was then neutralized with 0.01 N KOH solution in slight excess, dissolved at 75°C and adjusted with isotonic medium to the concentration of 0.04 mM and pH 7.4. Aliquots of this solution were used for incubation with blood cells; a new solution of palmitate-1-C^{14} was prepared for each experiment.

Incubation of blood cells. Blood cells were incubated with palmitate-1-C^{14} according to the same basic pattern as previously described (12). The incubation was done in a series of Warburg-type flasks, into the center wells of which small plastic cups containing 10% KOH and a small filter paper were inserted in order to capture the evolved $C^{14}O_2$. The incubation was terminated by addition of 0.1 N sulfuric acid into the main compartment of the incubation flasks, which also expelled the remaining $C^{14}O_2$ from the solution. Half an hour after the addition of sulfuric acid, the incubation flasks were opened, the plastic cups containing 10% KOH with the evolved $C^{14}O_2$ were removed and $BaC^{14}O_3$, precipitated with an excess of 1 M $BaCl_2$, was recovered and assayed for radioactivity. Isotope measurements were carried out with an Automatic Scaler Unit, model 183A, from Nuclear Chicago Corp.; the background was about 16 counts per minute. Radioactivity was usually measured as the time required for 25,600 counts. Counts were corrected for self-absorption to infinite thickness(13).

Results are expressed as micromoles of palmitate-1-C^{14} converted to $C^{14}O_2$ during a 60-minute interval of incubation.† However, be-

† The percentage conversion of added substrate to $C^{14}O_2$ was calculated from the equation:

$$\frac{\text{(corrected c.p.m. of recovered } C^{14}O_2 \times \text{mM } BaCO_3) \times 100}{\text{(corrected c.p.m. of added substrate} \times \text{mM } BaCO_3)} = \text{percentage conversion}$$

This percentage was used to estimate the amount of palmitate-1-C^{14} converted to $C^{14}O_2$ by multiplying the micromoles of palmitate-1-C^{14} added per vessel by the percentage converted.

TABLE I. Oxidation of 0.01 mM Palmitate-1-C^{14} by Whole Blood in Increasing Dilutions.*

Volume of whole blood in cu mm per one flask			800	400	200	100	50	25	12.5	6.25
Degree of dilution of whole blood in each flask†			5	10	20	40	80	160	320	640
10^{-6} micromoles of palmitate-1-C^{14} converted to $C^{14}O_2$‡	per flask	range of values	154–182	160–192	153–189	126–158	95–119	67–85	44–58	27–39
		mean §	168	176	171	142	107	76	51	33
	per cu mm of blood	mean	.21	.44	.85	1.42	2.14	3.04	4.08	5.28

* Incubation time 60 min.
† Total volume of incubation mixture (whole blood and isotonic medium) in each flask is 4.0 ml.
‡ Data represent difference between experimental and blood-free control.
§ Range and mean values of 9 studies.

cause the concentration of the added palmitate-1-C^{14} was chosen as low as 0.01 millimolar (0.26 mg/100 ml) in order to reduce to a minimum any possible modification of the cell metabolism by the added substrate, its amount represents only a fraction of the total amount of unlabeled substrates already present exogenously in blood plasma or endogenously in blood cells and available for their metabolic purposes. Since the appearance of $C^{14}O_2$ is the resultant of a series of enzymatic reactions and since any unlabeled substrate that contributes to the final common acetyl-CoA pool dilutes the collected $C^{14}O_2$, the converted palmitate-1-C^{14} represents only a small fraction of the total caloric expenditure of the studied cells. Nevertheless, the conversion rates of palmitate-1-C^{14} to $C^{14}O_2$ demonstrate exactly the changes in rates of substrate oxidation in each flask induced by changes of the number of white cells, platelets and red cells in various cell populations.

There is a constant and relatively very small non-enzymatic evolution of $C^{14}O_2$ from palmitate-1-C^{14}(14), but no additional increase in $C^{14}O_2$ evolution was detectable upon incubation of palmitate-1-C^{14} with cell-free blood plasma. Data of converted palmitate-1-C^{14} in Tables I and II represent the difference between experimental and blood cell-free control.

Results. During preliminary experiments, some of which were done with human blood (6), it was observed that the rate of $C^{14}O_2$ evolution from palmitate-1-C^{14} by any of the 3 cellular elements of the blood (whether white cells, platelets or red cells) is very low in the presence of undiluted blood plasma, but its increase after washing the cells in isotonic medium is different for each cell type, being about 60- to 90-fold for platelets, about 20- to 30-fold for white cells of the buffy coat and still lower for red cells. These differences, due in great part to unequal washing which cannot be avoided whenever washing of various populations of blood cells is done, make it very difficult to calculate and compare exactly the rates of substrate oxidation by these cellular elements of the blood. Later, however, it was observed that the dilution of the whole blood by isotonic medium increases the rate of palmitate-1-C^{14} oxidation by blood cells. Data of Table I are typical of these observations.

Table I presents data on $C^{14}O_2$ evolution by decreasing volumes of whole blood incubated for 60 minutes with 0.01 millimolar palmitate-1-C^{14} in a series of Warburg-type incubation flasks, each time adjusted to the same final volume by addition of isotonic medium. It can be seen that the amount of converted palmitate-1-C^{14} per cu mm of whole blood increases with each increasing dilution of the blood. It is after this observation, the detailed mechanism of which is still being studied, that most of the investigations on the relative rates of palmitate-1-C^{14} oxidation by blood cells were continued with the whole blood diluted 10 times, or with populations of blood cells suspended in blood plasma of the same dilution.

Table II presents data on $C^{14}O_2$ evolution from 0.01 millimolar palmitate-1-C^{14} dur-

TABLE II. Oxidation of 0.01 mM Palmitate-1-C^{14} by 10-Times Diluted Whole Blood and by 3 Blood Cell Populations Separated by Differential Centrifugation from Another Aliquot Volume of the Same Diluted Blood.*

Sample		No. of cells per cu mm			10^{-6} micromoles of palmitate-1-C^{14} converted to $C^{14}O_2$ per one flask¶
		White cells	Platelets	Red cells	
0.4 ml of whole blood diluted 10 times†	Range of values	8,700–11,200	740,000–900,000	8,400,000–9,800,000	158–186
	Mean§	9,950	820,000	9,100,000	172
1st cell population‡ (platelets-rich plasma)	Range of values	‖	610,000–720,000	‖	45–57
	Mean§		665,000		51
2nd cell population‡ ("buffy coat" with white cells)	Range of values	8,400–10,100	85,000–130,000	2,100,000–2,700,000	58–75
	Mean§	9,250	107,500	2,350,000	66
3rd cell population‡ (red cells from the bottom of the centrifuge tube)	Range of values	400–900	35,000–50,000	6,100,000–7,400,000	40–53
	Mean§	650	42,500	6,750,000	46.5

* Incubation time 60 min.

† Counts per cu mm of undiluted blood of which 400 cu mm were incubated in isotonic medium with palmitate-1-C^{14} in the total volume of 4.0 ml in one flask.

‡ Each blood cell population was resuspended in blood plasma of the same dilution and adjusted to the original volume of 10-times diluted blood from which these cells were separated.

§ Range and mean values of 10 studies.

‖ Platelets-rich plasma contained no detectable white cells or red cells.

¶ Data represent difference between experimental and blood cell-free controls. Percentage conversion of palmitate-1-C^{14} to $C^{14}O_2$ can be calculated by dividing micromoles converted by micromoles added (0.04 micromole per flask). For details see text.

ing 60 minutes of incubation with 4.0 ml of 10-times diluted whole blood or with 4.0 ml of each of 3 different blood cell populations suspended in blood plasma of the same dilution. The 10-times dilution of the whole blood resulted from addition of 1.0 ml of 0.04 millimolar palmitate-1-C^{14} to 3.0 ml of 7.5-times diluted blood, or to blood plasma of the same dilution containing various blood cell populations which were separated by differential centrifugation. The separation of the cell populations was performed in such a way that the total amount of white cells, platelets and red cells present in 10 cu mm from each of these 3 populations together was equal to the count of white cells, platelets and red cells present in 10 cu mm of 10-times diluted whole blood.

Comparison of data from the first line of Table II, obtained with diluted whole blood, to data from the second and third lines obtained with platelets-rich plasma and with white cells of the "buffy coat," indicates that white cells and platelets account for the major part of $C^{14}O_2$ evolution from palmitate-1-C^{14} by whole blood cells, whereas the portion of fatty acid oxidation attributable to the red cells is relatively very small, as may be seen from data in the fourth line of Table II. With the mean values from the second, third and fourth lines of this Table, 3 equations with 3 unknowns can be construed, from which the rate of palmitate-1-C^{14} oxidation of pure white cells, pure platelets and red cells can be calculated. Thus, from the second line, the mean amount of palmitate-1-C^{14} converted to $C^{14}O_2$ by 10^6 platelets is equal to 76.7×10^{-6} micromoles, whereas that by 10^6 white cells is $4{,}740 \times 10^{-6}$ micromoles and that by 10^6 red cells is 5.94×10^{-6} micromoles, as calculated from the third and fourth lines of Table II. Comparison of these values indicates that the rate of palmitate-1-C^{14} conversion to $C^{14}O_2$ by a given amount of white cells is about 60 times greater than that by the same amount of platelets, and about 800 times greater than that by the same amount of red cells. However, this last value is based on data obtained by red cells together with reticulocytes, the youngest circulating cells which, in the rat, amount to about 3 to 4% of total red cell count and

which themselves may well account for the major part and probably all of the $C^{14}O_2$ evolved from palmitate-1-C^{14} by red cells.

Discussion. The present investigation indicates that blood cells vary considerably in their capacity for the oxidation of fatty acids *in vitro* and that white cells and platelets account for the major part of $C^{14}O_2$ evolution from palmitate-1-C^{14} by whole blood cells. On a per cell basis, rate of fatty acid oxidation is about 60 times greater in white cells than in platelets and about 800 times greater than in red cells with reticulocytes. However, owing to the greater number of platelets in a volume of whole blood, 35 to 40% of total fatty acid oxidation by whole blood cells may be attributed to platelets. The portion of fatty acid oxidation attributable to the sum of red cells with reticulocytes, which may amount to about 25 to 30% of total fatty acid oxidation by whole blood cells, is probably covered entirely by reticulocytes alone. It has been repeatedly demonstrated that the reticulocyte has an intact Krebs tricarboxylic acid cycle, whereas in the mature red cell this cycle is no longer intact(15) or does not function(16), so that the mature red cell derives its energy primarily from anaerobic glycolysis and from oxidation of glucose *via* the hexosemonophosphate pathway(21). Also in accordance with the participation of reticulocytes in fatty acid oxidation *in vitro* is the observation that the rate of palmitate-1-C^{14} oxidation by washed whole blood cells from young rats is higher than the rate by whole blood cells from adult rats(17), which in great part can be explained by the higher percentage of reticulocytes in the blood of young rats(18).

The rate of palmitate-1-C^{14} oxidation by blood cells is much lower in undiluted plasma, increases by washing in isotonic medium and decreases again by addition of blood plasma to the cells(6,12). This effect of blood plasma is due in part to the presence of glucose, non-esterified fatty acids and other metabolites, as well as to the effect of plasma albumin, which is known to bind non-esterified fatty acids in water-soluble complexes(19) and to decrease the rate of palmitate-1-C^{14} oxidation by blood cells(12) or by skeletal muscle *in vitro*(20). However, the inhibitory effect of plasma albumin on palmitate-1-C^{14} oxidation by blood cells is only in part due to the binding of non-esterified fatty acids, because other soluble proteins and also non-proteic, hydrophilic, macromolecular substances have a similar effect(10). The mechanism of action of these substances is being investigated.

Summary. Fatty acid oxidation by blood cells *in vitro* has been studied by incubation of 10-times diluted blood of the rat with palmitate-1-C^{14}, followed by determination of $C^{14}O_2$ evolved. A modification of the method was introduced for separation of white cells, platelets and red cells by differential centrifugation, in order to increase the accuracy of the determinations. The effect of diluted plasma on rate of palmitate-1-C^{14} oxidation and action of plasma albumin were taken into consideration. Experimental data indicate that a major portion of the total palmitate-1-C^{14} oxidation *in vitro* per unit volume of blood is attributable to white cells and platelets, whereas palmitate-1-C^{14} oxidation by red cells is very limited and probably occurs only in the youngest circulating cells. On a per cell basis, white cells oxidize palmitate-1-C^{14} at a rate which is about 60 times greater than that of platelets and about 800 times greater than that of red cells with reticulocytes.

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Experiences with Furosemide in Renal Disease. (29835)

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This paper presents data on the renal hemodynamic and diuretic effects of a new compound, furosemide.* This compound is an anthranilic acid derivative whose structure differs from that of the thiazides and ethacrynic acid (Fig. 1). A symposium held in Germany in 1963 (unpublished) furnished sufficient data to suggest that furosemide was a potent diuretic with relatively few side effects. Our particular interest has been an examination of its acute effects in patients with renal disease associated with various degrees of reduction in renal function.

Material and methods. a. Intravenous studies. Fifteen patients were selected from the wards of the Louisville General Hospital and the renal clinic. All but 3 were edematous. The patients ranged in age from 21 to 75 years and included 8 with the nephrotic stage of chronic glomerulonephritis, 2 with Kimmelstiel-Wilson's disease, and 5 with chronic pyelonephritis. Cirrhosis and congestive failure, respectively, had produced edema in 2 of these five. Two subjects were studied a second time after an interval of a few months and a change in renal function.

On the day of the study, the subjects drank a water load of 10-20 ml/kg and were suitably prepared with bladder catheters and infusions for para-aminohippurate (PAH), and other clearances. After a period of stabilization, two 30-minute periods were used to measure the clearances of endogenous creatinine, PAH, and in most cases, sodium, potassium, chloride, free water(1), and total osmols. At the end of the second control period, 40 mg of furosemide were injected intravenously and the measurements were repeated as above for three 30-minute periods.

Standard methods were employed for measurement of creatinine(2) and PAH(3). Sodium and potassium concentrations in blood and urine were measured by flame photometry; osmolality was determined by cryoscopy and chloride by the Cotlove chloridometer.

b. Oral studies. Six subjects (Table III) were studied using divided, daily doses of 160-640 mg. They were restricted in salt intake and were receiving no other diuretic drugs. Observations made included body weight, serum electrolytes, BUN, uric acid and peripheral blood counts. Twenty-four hour urines were collected and analyzed for sodium, potassium and chloride, but the collection problems on the general medical wards precluded calculations based on urine volumes. Four of the 6 subjects had also taken part in the intravenous studies.

Results of intravenous studies. a. Creat-

* Kindly furnished by Lloyd Brothers, Inc., Cincinnati, Ohio.