We have begun an investigation of the level of enzymatic activity in tissues which have been infiltrated by malignant cells. Two preliminary reports of this work have appeared (1, 2). We are presenting herewith studies of fatty acid oxidase activity which have been carried out on C58 mouse liver homogenates according to Lehninger (3, 4). In addition, results of orienting experiments with rat liver systems will be reported.

Since one aspect of the cancer problem is the study of the chemical reactions which take place in living tissue, and since all major reactions in vivo involve enzymatic influence, it is important to determine the effect which invading malignant cells may have on the catalytic apparatus of normal tissue. The majority of the studies of enzymes in relation to cancer have been similar to those described by Greenstein and co-workers (5) and have involved a comparison of the enzymatic levels of normal versus tumor tissue. It seems important to us to study organs which are more or less diffusely infiltrated by malignant cells. The C58 strain of mice developed by MacDowell offers excellent experimental material for this type of study, since numerous tissues in these animals are invaded by malignant lymphocytes as spontaneous lymphatic leukemia develops (6, 7). Histological examination of typical tissues from C58 mice shows general infiltration with pronounced concentrations of malignant cells around the periphery of blood vessels. Entire organs are attacked and are subjected to a competition for metabolites with the actively multiplying lymphocytes.

Using liver slices from leucemic mice (Rf and Rf/Ak strains) Burk et al. (8) have described experiments involving oxygen uptake, CO₂ production, and aerobic and anerobic glycolysis. Their results show, in general, elevated glycolysis (particularly in anerobic systems) in the case of infiltrated livers. Hall (9) has reported similar results in anerobic glycolysis studies on liver slices from leucemic Ak mice. We have confirmed these observations on anerobic glycolysis with C58 mouse liver slices (10). (For results of studies of the metabolism of lymph nodes from leucemic mice, see Victor and Potter (11).)

Our results can be summarized in advance by stating that in C58 mouse livers there is frequently observed a profound loss of fatty acid oxidase activity (as measured by the Lehninger technique) associated with leucemic infiltration. In such cases the extent of loss of activity usually greatly exceeds the extent of replacement of liver cells by malignant lymphocytes. Histological examination of the C58 mouse livers has been made in every case, and an estimate of the degree of infiltration obtained using Chalkley’s method (12). In our experiments whole homogenates and washed, mal-
onate-inhibited systems from infiltrated mouse livers showed essentially similar net fatty acid oxidase activities.

Lehninger (4) reported that rat liver fatty acid oxidase systems were susceptible to inhibition by excess substrate. We have made a study of the relationship between octanoate concentration and oxygen uptake, using whole rat liver homogenates and washed, malonate-inhibited rat liver homogenates. Both types of system show a peak of activity at 0.001 M octanoate concentration. Our mouse experiments have been carried out at this concentration.

**EXPERIMENTAL**

For the work to be described the following special materials were obtained as indicated: Cytochrome-c by the method of Keilin and Hartree (13) modified by final dialysis against distilled water and lyophilization, adenosine triphosphate according to LePage (14), octanoic acid (Eastman), a-ketoglutaric acid synthesized by E. D. Nielson in this laboratory according to Schneider (15), adenylic acid by hydrolysis of adenosine triphosphate.

Livers were homogenized for 2 to 3 minutes in two parts of ice-cold Krebs-Ringer phosphate buffer (pH = 7.7; calcium omitted) with the aid of a Potter-Elvehjem glass homogenizer (16). After removal from the animals the livers were immediately cooled in ice for 5 minutes before homogenizing. In the preparation of washed systems the whole homogenates were centrifuged at 0 to 5° for 5 minutes in a clinical centrifuge at 2500 r.p.m. and the supernatant fluid discarded. The centrifuged material was then washed with ice-cold Krebs-Ringer phosphate buffer in sufficient amount to bring the system to its original volume after centrifugation. The washing and centrifuging were done 3 times. After the final washing the system was made up to three-fourths of its original volume with the ice-cold buffer.

In our early experiments Lehninger's first technique (3) involving whole homogenates was used (17). It became apparent that further knowledge of the effects of varying substrate concentration was required. Hence, the oxygen uptake of rat liver systems as a function of octanoate concentration was studied. The results of an...
experiment of this type are shown in Figure 1. On this graph are also plotted results for a washed malonate-inhibited rat liver system. These results indicate that 0.001 M octanoate concentration permits optimal activity.

From experiments with washed, malonate-inhibited rat liver systems it was possible to plot the reciprocals of the initial velocities (15 minutes values of the oxygen uptake) against the reciprocals of the octanoate concentrations. This permits evaluation of the Michaelis constant ($K_m$) according to the slope-intercept form of the Michaelis equation: (18)

$$\frac{1}{v} = \frac{1}{V_{max}} \cdot S + \frac{1}{V_{max}}.$$

In this equation $v$ is the observed initial velocity, $V_{max}$ is the graphically estimated limiting velocity, and $S$ is substrate molarity. The values of $K_m$ for 6 experiments with normal adult rat liver were 1.88, 1.64, 2.0, 1.71, 1.51, and $1.45 \times 10^{-4}$ M. The average $K_m$ value was $1.70 \times 10^{-4}$ M.

Lehninger's (19) and our results with the washed systems show a rapid near-quantitative transformation of octanoate to acetoacetate under the conditions specified in Figure 1. The oxygen uptake approaches the theoretical amount required for the reaction:

$$\text{CH}_3 \text{(CH}_2)_\text{6COOH} + 3\text{O}_2 \rightarrow 2\text{CH}_2\text{COCO}_2\text{COOH} + 2\text{H}_2\text{O}$$

Chemical analysis for acetoacetic acid produced in the reaction by Edson's method (25) amply confirms Lehninger's results. An indication of the reproducibility of the results encountered in the rat liver systems may be obtained from the following: Experiments with 15 normal rat livers from stock animals of various ages and both sexes showed an average oxygen uptake in 45 minutes of 48 ± 8 μl. This corresponds to 72 ± 12 per cent of the theoretical activity and was checked in several cases by acetoacetate analysis.

Essentially similar results were obtained with C58 mouse liver systems prepared from animals in which there was very little or no infiltration by malignant lymphocytes. In Figure 2 are presented the results of a typical experiment with a non-leukemic (or pre-leukemic) C58 animal. Similar activities were encountered with the livers from mice of other strains (C57 black and Zr).¹ The average oxygen uptake for two C57 animals and one Zr animal studied according to these exact conditions was 53 ± 7 μl in 45 minutes.

It should be emphasized at this point that a major factor involved in the reproducibility of results appears to be homogenization with a rather loose-fitting homogenizer for short periods of time. We adopted a rigid time schedule of operations in handling the livers. Whenever a system of low activity was encountered, a normal rat liver was tested with the same reagents in order to make sure that all solutions were properly constituted.

In Figure 3 are shown the results of an experiment with a severely infiltrated C58 mouse liver. In this case there was substantially no fatty acid oxidase activity. Other leukemic animals showed intermediate levels of oxidase activity.

In Figure 4 are presented data from experiments designed to examine the relation between oxygen uptake and octanoate concentration in the case of three C58 mice: one a non-leukemic animal and the other two decidedly leukemic. It is apparent from these and similar experiments that 0.001 M octanoate is a favorable concentration for a comparison between non-infiltrated and infiltrated livers. It was not possible to obtain reliable estimates of the Michaelis constant in the case of severely infiltrated mouse livers.

The results of fatty acid oxidase studies on thirty-two C58 mice are summarized in Table 1. In this table are included data from experiments with washed, malonate-inhibited systems only. Substantially similar results were obtained with whole homogenates. The per cent conversion of octanoate to acetoacetate was calculated from the oxygen uptake after 85 minutes in each case, at which time the systems had practically stopped absorbing oxygen. The mouse numbers are those of MacDowell.

The per cent infiltration values must be regarded as estimates only. Two randomly selected pieces of liver from each animal were removed when the animal was sacrificed. These were fixed in alcohol-formalin-acetic acid, imbedded in paraffin, sectioned and stained with eosin-hematoxylin.² The sections were evaluated using Chalkley's (12) technique: We have as a rule examined 20 fields using an ocular equipped with 5 pointers, and have found that the results are sufficiently accurate for our purposes, and quite well reproduced in independent counts made by different observers. In this way the numbers recorded in Table 1 were obtained. We feel that we have arrived at reasonable approximations of the extent of infiltration, even though in some animals the infiltration was rather spotty.

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²The authors wish to thank Dr. Eldon D. Nielsen and Ursula Irish for valuable assistance with the histological work.
There is a good correlation of spleen size with the severity of the leucemic state of C58 animals (Table I). We have weighed the spleens in every case and considered spleens weighing less than 0.2 gm. to be normal or pre-leucemic. As described by MacDowell, the leucemic C58 animal frequently exhibits spectacular enlargement of liver, thymus, spleen and lymph nodes. In a few cases fatty livers were found, and in all of these cases fatty acid oxidase activity was very low. A few of the leucemic animals were severely emaciated with almost complete loss of body fat. Several of these animals showed fatty livers. The majority of the leucemic animals possessed reasonably active appetites at the time of sacrifice, so that our results cannot be ascribed to inanition (20).

From the results in Table 1 we conclude that there is often a failure of systems from infiltrated livers to attack octanoate. Inspection of the data obtained from 92 animals of varying leucemic state reveals that 19 of them showed less than 50 per cent of the theoretical fatty acid oxidase activity. Of these 19, only 5 animals showed malignant cell infiltrations higher than 10 per cent, and 13 animals showed less than 20 per cent of the theoretical oxidase activity. Of the 18 mice exhibiting more than 50 per cent of the theoretical fatty acid oxidase activity, 10 fall in the range, 80 to 100 per cent activity, and of these only one (8 per cent infiltration) showed an infiltration by malignant cells greater than 3 per cent. Thus it appears that certain of these animals show effects of leucemic infiltration to a greater extent than others. Some of the variability in our results is considered to be due to the fact that we were encountering the consequences of malignant cell invasions of widely varying duration. Variations in results from mice with low degrees of liver infiltration also suggest the possibility that the severity of the leucemic state may not always be accurately indicated by the extent of malignant cell invasion. In the case of No. 82646 a low infiltration was associated with a large spleen. Animals 93501, 79365, 93529 and 75779 showed somewhat enlarged spleens, but small amount of infiltration and substantially normal fatty acid oxidase activity. The significance of the grossly fatty livers encountered in several animals is not understood.
DISCUSSION

Since fatty acid oxidase activity is known to be associated with the sedimenting particles in a liver homogenate (21, 22), it seemed of interest to compare systems homogenized in Kребs-Ringer phosphate with those homogenized in hypertonic 0.88 M sucrose, which medium tends to preserve particulate structure. No difference in fatty acid oxidase performance was observed in the case of a severely infiltrated mouse liver when it was homogenized in either medium. Potter (28) has shown that homogenization of rat liver in hypotonic medium leads to loss of fatty acid oxidase activity. This loss may be considered to be associated with the disorganization of certain subcellular elements. Potter (24) has suggested that the "cytolysis quotient" is an index to the integrity of certain intracellular entities. The term, "cytochrome quotient," might be applied to suggest the possibility that homogenization in hypotonic medium leads to the dissociation of cytochrome from certain protein surfaces. In our studies homogenization of livers from a series of mice of varying leucemic state showed no change of "cytochrome quotient" as a function of degree of infiltration (10). Accordingly our fatty acid oxidase results are not readily explained on the basis of fragility of intra-cellular elements, but seem to suggest an actual deficiency of enzymatic surface.

In a few experiments with C58 mice α-ketoglutarate and adenylic acid were substituted for ATP (4). Similar results with infiltrated livers were obtained when this modification was employed.

In one experiment equal amounts of washed normal mouse liver (Zr strain) and of highly infiltrated C58 mouse liver were used in an attempt to detect any possible inhibitor effect. No such effect was observed. The Zr mouse liver system and the mixed system showed a near-quantitative conversion of octanoate to acetoacetate, but the C58 leucemic mouse liver system was almost wholly inactive.

An additional point needs to be made. Since we are studying the enzymatic activity of liver "systems" of variable malignant lymphocyte content, we may get a picture of some of the enzymatic capabilities of lymphocytes in cases of severe infiltration. However, in those cases where the infiltration is low, it is our opinion that we are dealing with lowered activity of the liver cells themselves.

How far these results may be considered to bear upon the situation existing in the intact animals is difficult to state. We feel that the best interpretation of the data at present is based upon the concept of a competition for metabolites in an invaded organ between the rapidly multiplying malignant lymphocytes and the liver cells. This competition favors the lymphocytes, in part because of their concentration around the periphery of blood vessels. The net result is a deficiency of certain intracellular proteins (enzymes). In support of this idea is the fact that our studies showed in several cases a sharp loss of fatty acid oxidase activity in livers which were infiltrated to only a small extent (less than 10 per cent).

SUMMARY

1. The oxidation of octanoate in normal rat liver and in C58 mouse liver homogenates has been studied.
2. Data from thirty-two C58 animals of varying leucemic state have been presented. Nineteen of these animals showed fatty acid oxidase activ-
ities less than 50 per cent of the theoretical. Thirteen of these 19 animals yielded homogenates exhibiting less than 20 per cent of the calculated activity.

3. A discussion of possible implications of these results is presented.

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The Oxidation of Octanoate by Liver Homogenates from Leucemic Mice

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