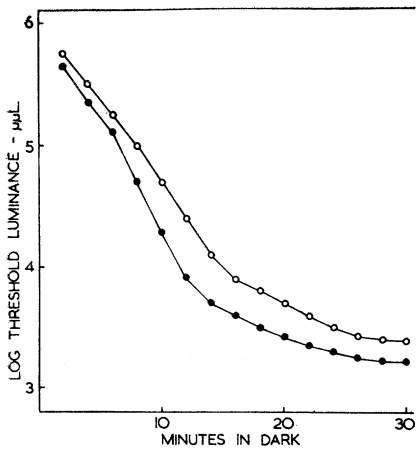

Fig. 1. Mean dark-adaptation curves: ○, psychiatric patients; ●, normal subjects (see *2*).

perimental analysis of the responses of different psychiatric categories may reveal at least statistically significant differences between certain diagnostic groups, not only with respect to the position of the dark-adaptation curve on the intensity axis, but also with respect to its shape. Observations in two experiments (*11*) suggest, for instance, that hysterics and anxiety states may differ in the level of their dark-adaptation curves, and one or two hysterics have produced curves that differ in slope from the normal. Such differences suggest that, at least in certain cases, the mechanism of dark adaptation may be impaired, and detailed investigation of these cases may be of considerable interest from the viewpoint of visual research as well as psychiatry. It is possible, for instance, that neural mechanisms operating in dark adaptation may be affected, perhaps via the centrifugal fibers known to exist in the optic nerve. In view of recent work on the "reticular activating system," both in regard to centrifugal control of the sense organs and its role in psychiatric disorder (*12*), such a possibility cannot be dismissed. Worth noting also is the further possibility that differences in dark-adaptation thresholds between anxiety states and hysterics may be paralleled by differences observed between normal subjects classified according to temperamental type (*13*).

With regard to the experimental implications of the general hypothesis suggested in a preceding paragraph, one might expect, on the basis of previous psychophysical research on vision, that changes would occur in the curves that relate a number of visual functions other than absolute thresholds to intensity. Thus, one would expect the curves that relate critical flicker frequency, brightness discrimination, and visual acuity also to be shifted along the intensity axis by psychiatric disorder. The extent of the shift should in each case be similar to the amount of displacement of the dark-adaptation curve, since it is a function of the density of the hypothetical filter that was assumed to have been placed in front of psychiatric patients' eyes. These "deductions" are all susceptible to experimental test.

One final point that requires emphasis concerns the magnitude of the differences so far obtained that can be attributed to the effects of psychiatric disorder. As was pointed out in a preceding paragraph, in certain cases the difference amounts to as much as 0.5 $\log_{10}$ unit, but in other cases it may be as little as 0.1 $\log_{10}$ unit. Although the differences were statistically significant under the experimental conditions in which they were observed, they must be related to the whole body of knowledge concerning the visual mechanism if they are to be seen in proper perspective. Compared with the enormous range of values (1 to 10^6) through which the visual threshold can vary during light and dark adaptation, the changes are very small indeed. Because it seems likely from studies of physiological stresses (*14*) that the observed changes represent the limits of variation that can be expected from disturbances central to the photochemical system, it is clear that refined experimental techniques will be necessary to measure them accurately and to assess their significance in relation to physical and other sources of variation, including individual day-to-day variation, which is often of the order of 0.3 $\log_{10}$ unit (*15*).

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19 December 1956

Histochemical Distribution Pattern of Respiratory Enzymes in the Liver Lobule

Knowledge concerning the function and distribution of enzymes has been gained primarily from procedures involving the structural disintegration of tissue. However, recently developed methods have permitted the localization of specific enzymes in tissue sections. This study deals with the histochemical distribution pattern of four respiratory enzymes—succinic dehydrogenase, cytochrome oxidase, diphosphopyridine nucleotide diaphorase (DPN-diaphorase), and triphosphopyridine nucleotide diaphorase (TPN-diaphorase), respectively—in the liver lobule of higher mammals.

The enzymes concerned are intimately linked to the tricarboxylic acid cycle, which is believed to be the major pathway for the oxidation of many metabolites. Hence, the histochemical study of these enzymes may provide some general information concerning the structural organization of tissue respiration. Furthermore, it may help to elucidate the biochemical anatomy of the liver and its possible significance in the development of localized lesions in liver injury.

The histochemical demonstration of the succinic dehydrogenase system (*1, 2*) and of the DPN- and TPN-diaphorases (*3*) is based on the enzymatic reduction of tetrazolium salts, which serve as indicators of local reductase activity. On reduction, the water-soluble, colorless tetrazolium is converted into a water-insoluble, colored formazan at the site of enzyme activity. The intensity of staining appears to give a rough indication of the amount of enzyme activity present. To achieve specificity of the individual assay, tissue sections are incubated in a medium that allows only the particular enzyme under study to operate on the reduction of the added tetrazolium salt. These methods satisfy most of the criteria that have been adopted for the specificity and reliability of histochemical stains, and they are thoroughly discussed by Farber, Sternberg, and Dunlap (*3*).

The cytochrome oxidase was demonstrated by means of the G-Nadi reaction (*4*), which is based on the oxidative reaction between α-naphthol and dimethyl-*p*-phenylenediamine to form indophenol blue.

In all experiments, frozen sections of freshly obtained pig, horse, and beef liver were incubated in the appropriate media for 30 to 120 minutes at 37.5°C and washed briefly; after formalin fixation, they were mounted in glycerin jelly. The sites of enzyme activity were demonstrated by the more or less intensive deposits of dark blue formazan or indigo blue particles, respectively.

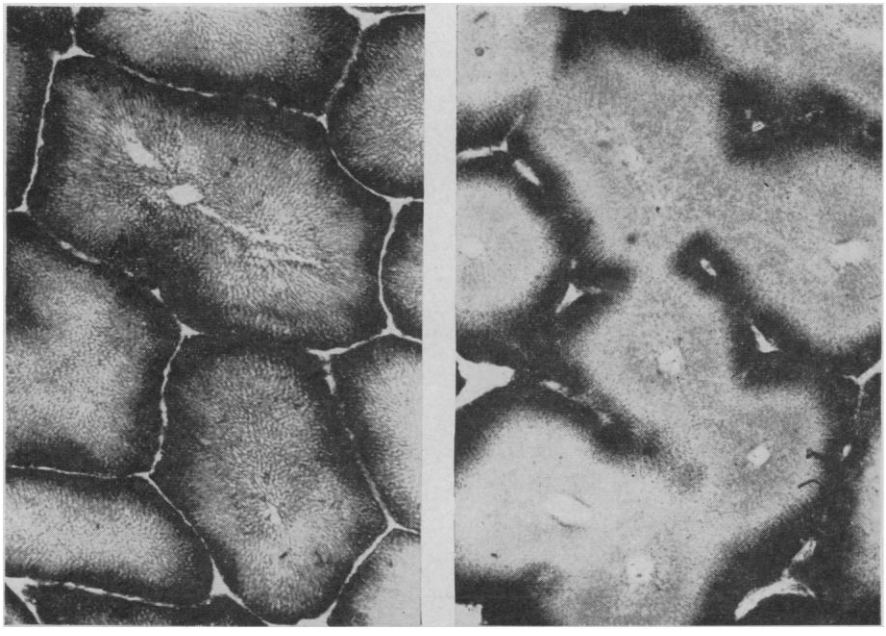

Fig. 1. Distribution of succinic dehydrogenase (dark areas) in the pig (left) and horse (right) liver. Note peripheral concentration of enzyme activity in both species, showing segmental interruptions at the nodal points in the horse. The incubating medium consisted of 5.0 ml of sodium succinate (0.2*M*); 0.1 ml of $CaCl_2$ (0.33*M*); 0.4 ml of $AlCl_3$ (0.01*M*); 0.1 ml of $MgSO_4$ (0.005*M*); 1.0 ml of $NaHCO_3$ (0.6*M*); 5 ml of neotetrazolium (2.0 mg/ml); 0.5 ml of KCN (2-percent); 4.5 ml of phosphate buffer (0.2*M*) at *p*H 7.6; and 3.4 ml of water.

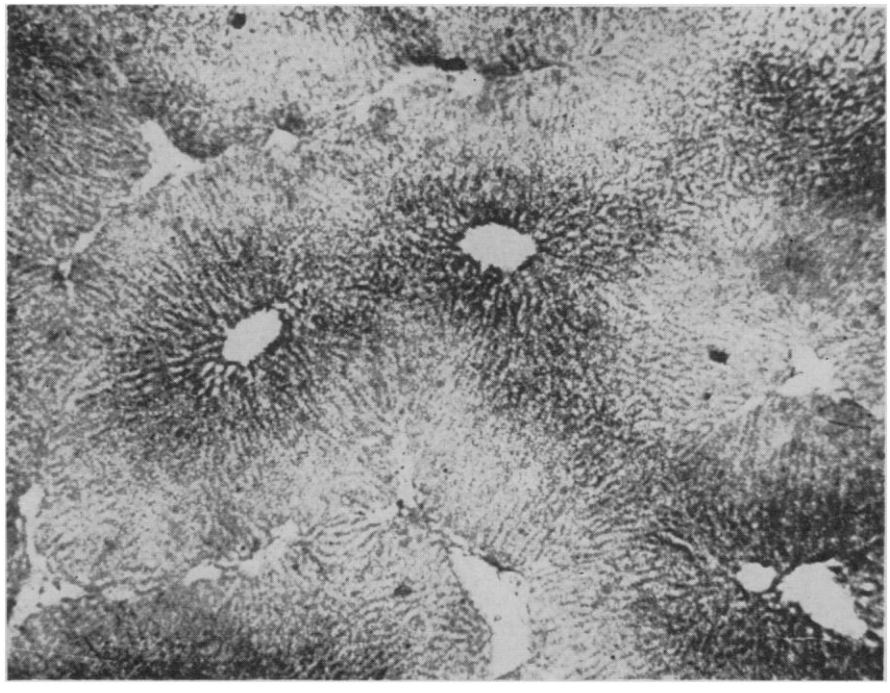

Fig. 2. Distribution of DPN-diaphorase (dark areas) in the horse liver. Note the concentration of enzyme activity around central vein areas, extending segmentally toward the nodal points. The incubating medium consisted of 0.1 ml of alcohol dehydrogenase (1.5 mg/ml); 0.2 ml of ethanol (1.09*M*); 0.3 ml of sodium L-malate (0.5*M*); 0.5 ml of sodium L-glutamate (0.5*M*); 0.2 ml of DPN (5.0 mg/ml); 0.2 ml of semicarbazide (0.1*M* in PO_4 buffer); 0.7 ml of blue tetrazolium (1.0 mg/ml); and 0.8 ml of phosphate buffer (0.1*M*) at *p*H 7.4.

The following results were obtained. (i) The staining for each of the respiratory enzymes showed a highly distinctive and characteristic zonal distribution pattern in the liver lobule. (ii) Succinic dehydrogenase and cytochrome oxidase were particularly concentrated in the periphery of the lobule, their density decreasing rapidly toward the center of the lobule (Fig. 1). (iii) The diaphorases exhibited an almost reverse pattern of localization, showing maximal concentration in the center and more (DPN-diaphorase) or less (TPN-diaphorase) rapidly decreasing density toward the periphery of the lobule (Fig. 2). (iv) Segmental variations of the zonal distribution pattern in different animals are related to the anatomical structure of the liver lobule in these species (Fig. 1).

On the basis of the afore-mentioned assumptions concerning the reliability of histochemical stains, the present results indicate zonal and segmental differences in enzyme activity, which are closely related to the blood flow in the liver lobule. Since the blood enters the lobule at the periphery and escapes through the central vein, there is a corresponding decrease of oxygen tension from the periphery of the lobule to its center. The enzyme distribution obviously reflects this relationship; the concentration gradients of cytochrome oxidase and succinic dehydrogenase directly parallel the slope of oxygen tension, while both the DPN- and TPN-diaphorases exhibit almost reverse concentration gradients. This relationship is particularly striking at the nodal points of the lobule and requires further explication.

The blood is supplied to the lobule from terminal branches of the portal vein and the hepatic artery. Both tributaries join in the formation of a capillary network, which spreads more or less extensively at the periphery of the lobule and finally drains into the sinusoids. In the pig liver, this capillary network entirely surrounds the lobule, thus providing a rather homogeneous blood supply to each segment of the lobule. Hence, the oxygen tension decreases almost concentrically toward the central vein, thus promoting an almost zonal pattern of enzyme distribution (Fig. 1, left).

In the liver of horse, beef, and man, however, the capillary network is more or less confined to the vicinity of the larger tributaries at the edges of the lobule—that is, to the portal areas. Accordingly, the blood supply to the lobule favors the vicinity of the portal areas and

spares an area midway between two portal areas, which is far removed from any rapid and straight running circulation. Mall (*5*) termed these areas *nodal points*. The oxygen tension at the nodal points is almost as low as that around the central vein. Accordingly, the enzyme pattern at these sites corresponds to that of the centrolobular area, thus promoting characteristic segmental deviations of the zonal distribution pattern (Figs. 1, right, and 2).

Since the enzymes under study reflect only part of an integrated metabolic system, it may be assumed that other chemical components reveal similar distribution patterns in the lobule. Recent reports concerning the histochemical demonstration of glucose-6-phosphatase (*6*), adenosine triphosphatase (*7*), and aminooxidase (*8*) are consistent with this assumption.

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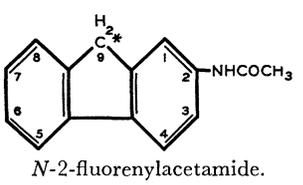
Orientation of Biochemical Hydroxylation in Aromatic Compounds

Recent reports (*1*) suggest the existence of different mechanisms for the *ortho* and *para* hydroxylation, respectively, of aromatic ring systems. Further evidence on this point can be derived from the following studies. Experiments on the metabolism of *N*-2-fluorenylacetamide in the rat, a species in which this



N-2-fluorenylacetamide.

compound is carcinogenic, have established that hydroxylation occurs at the 1, 3, 5, 7, and 8 positions (*2*). Similar methods have now been used to identify the metabolites of this compound in the guinea pig, a species in which the material failed to induce tumors (*3*).

The data of Table 1, as well as the results of other metabolic experiments (*4*), show that hydroxylation by the guinea pig takes place to a large extent at the 7 position and in an insignificant amount at positions 1, 3, 5, and 8 of the fluorene ring system. This species difference might indicate that hydroxylation at the 1, 3, and 5 positions is carried out by one enzyme system (or perhaps several systems of one type), while hydroxylation at the 7 and 8 positions could be performed by a different mechanism. Apparently the guinea pig possesses, at best, minor amounts of the former, while the rat is endowed with appreciable quantities of both.

The 1 and 3 positions are *ortho* to the substituent in the 2 position and, thus, are subject to the action of an *ortho*-hydroxylase. The 5 position is, of course, remote from the substituent in the 2 position, but it can be considered to be *ortho* to the carbon-carbon bond linking the two phenyl rings of fluorene. Thus, this position might be susceptible to hydroxylation by an enzyme system causing oxidation at the *ortho* carbon atoms. The 7 carbon atom is in an extended *para* relationship to the substituent in the 2 position (*5*). Both the guinea pig and the rat produce a relatively large amount of the 7-hydroxy derivative but only a minor amount of the 8-hydroxy derivative in the metabolism of *N*-2-fluorenylacetamide. Both compounds could conceivably originate from a common intermediate, such as the corresponding dihydrodiol, by a nonselective dehydration favoring the 7-hydroxy derivative. These data are consistent with a hypothesis that a "*para*-hydroxylase" introduces hydroxy groups into aromatic compounds via such intermediates, whereas an *ortho*-hydroxylase may do so by a different mechanism.

These results may have bearing on studies dealing with the mechanism of carcinogenesis, for *ortho*-hydroxylation of certain aromatic amines has been postulated as being involved in the carcinogenic action of these amines (*6*). The production of the *ortho*-hydroxylated derivatives of *N*-2-fluorenylacetamide in the rat (a susceptible species) and the absence of these derivatives in the guinea pig (a resistant species) lends some additional support to that hypothesis.

Table 1. Paper chromatographic identification of metabolites of *N*-2-fluorenylacetamide in rat and guinea-pig urine. Urine was collected from rats and guinea pigs that had been administered *N*-2-fluoren-9-C^{14}-ylacetamide at a dosage of 10 mg/100 g of body weight. Ether extraction of the urines to remove nonconjugated compounds was followed by incubation with β-glucuronidase to hydrolyze the glucuronic acid conjugates. The hydrolyzed mixture was extracted again with ether. This ether extract was chromatographed on S and S paper No. 598 that had been cut in the machine direction in solvent system 3 (*2*), composed of cyclohexane, *t*-butanol, acetic acid, and water (16/4/2/1 parts by volume). The spots were revealed by autoradiography on film. The 8-hydroxy derivative cannot be seen on the chromatogram of rat urine extract because of tailing from the 5-hydroxy derivative (*2*). The pattern of the free, or nonconjugated, compounds was identical with that of the glucuronic acid conjugates (*4*).

Compound	Range of R_f values (× 100) (front to back of spot)	
	Rat	Guinea pig
N-(1-Hydroxy-2-fluorenyl)acetamide	66–88	
N-(3-Hydroxy-2-fluorenyl)acetamide	44–58	
N-(5-Hydroxy-2-fluorenyl)acetamide	18–29	
N-(8-Hydroxy-2-fluorenyl)acetamide	?	17–23
N-(7-Hydroxy-2-fluorenyl)acetamide	8–14	6–17

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6 December 1956