creased amount of radioactive iodine recoverable from the urine in a 24-hour period (38%) over that found in the urine of the normal patient (8-12%).

Summary. 1. Plasma volumes can be accurately determined by the radioactive (I-131) iodinated plasma protein method in nutritional hypoproteinemia. 2. The rate of disappearance of radioactive protein from the blood stream is the same in undernourished states as in normal and therefore cannot be utilized to determine the state of the protein reserves of the body. (3). In patients who are losing abnormal amounts of protein in the urine or into abscess cavities, the rate of disappearance appears to be increased. The use of radioactive serum albumin may offer a diagnostic aid in the detection of unexplained protein losses.

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In vivo Staining of Fat in Tumor Bearing Mice by Benzo[a]phenoxazine Dyes.* (19086)

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In a previous study(1) of the effects of the oral administration of dyes on tumor bearing mice it was found that certain benzo[a]phenoxazines containing 5-phenylamino groups stained fat *in vivo*. The N-phenyl derivative of Nile Blue A stained fat a brilliant orange and tumor tissue a pale blue color. On the other hand, it was shown that the 5-amino and 5-benzylamino - 9 - diethylaminobenzophenoxazines, Nile Blue A and Nile Blue 2B, respectively, stained tumor tissue deep blue but failed to stain fat *in vivo*. The failure of the 5-amino and the 5-benzylamino-9-diethylaminobenzophenoxazines to stain fat *in vivo* was of interest because Thorpe(2), Smith(3), and Heidenhain(4) had shown that these compounds stain fat *in vitro*. The *in vitro* differential fat staining effect was supposed to depend to some extent upon the fat solubility of the dyes or their chemical transformation products. Hadjioloff(5), however, in his review of the publications on *in vivo* staining of fat administered by the enteric route, showed that many fat soluble dyes failed to stain fat *in vivo*. In another study of the benzo[a]-phenoxazine dyes(6) it was found that of the dyes available at that time only those containing the 5-phenylamino group stained fat *in vivo*.

The present study is concerned with *in vivo* staining of fat in tumor bearing mice following oral administration of a number of substituted 5,9-diaminobenzo[a]phenoxazine dyes. The majority of these contained a 5-phenylamino group while the others had benzyl-, naphthyl- or heterocyclic amino groups in the 5-position. Generally, the hydrogens of the 9-amino group were replaced by alkyl radicals containing from one to 6 carbon atoms.

Material and method. Mice of inbred stains and tumors which were 100% transplantable in mice of the strain of the host

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1. Lewis, M. R., Goland, P. P., and Sloviter, H. A., *Anat. Rec.*, 1946, v96, 201.

2. Thorpe, J. F., *J. Chem. Soc.*, 1907, v91, 324.

3. Smith, J. L., *J. Path. and Bact.*, 1908, v12, 1.

4. Heidenhain, Martin, *Arch. ges. Physiol.*, 1902, v90, 115.

5. Hadjioloff, A., *Bull. d'histol.*, 1938, v13, 81.

6. Lewis, M. R., Goland, P. P., and Sloviter, H. A., *Cancer Research*, 1949, v9, 736.

were utilized in these studies. A few tumor bearing rats were also used for a comparison of the staining behavior of animals of a different species. The dyes to be tested were ground together with pulverized Purina fox chow in concentrations of 0.2% with the exception of 5-benzylamino-9-dibutylamino, 5-(4-chlorobenzylamino)-9-dibutylamino and 5-(4-methylbenzylamino) - 9-dibutylamino-benzo[a]phenoxazonium chlorides, which were prepared in 0.05% concentrations. The mixtures were fed to the animals for 15 days, beginning on the day the tumor tissue was implanted. At the end of this period, the animals were sacrificed and the results observed. Generally the dyes were used in the form of their salts, such as their chlorides or nitrates, although in a number of instances the dye bases were administered. The preparation of these compounds has recently been described(7).

Results. In these investigations of the effect of benzo[a]phenoxazine dyes on fats and tumors in mice, it was found that the majority of the 5-phenylaminobenzo[a]phenoxazine compounds used stained fat *in vivo* (Table IA). The exceptions were those containing hydroxyl and carboxyl groups on the phenyl nucleus. All of the dyes studied were 5,9-diamino derivatives. In most instances the substituents on the 9-amino nitrogen were alkyl groups, but in certain compounds the carbon chains were bridged by an oxygen atom, giving the morpholinyl radical. This ring structure in the 9-position did not change the behavior of the dyes in the *in vivo* staining of fats.

The phenyl radical substituted for one hydrogen atom of the 5-amino group decreased the *in vivo* tumor staining properties of the resulting dyes, while the benzyl radical increased these properties.

5-benzylamino-9-dimethylamino- and 5-benzylamino- 9 -diethylaminobenzo[a]phenoxazine dyes or their benzyl ring substitution products stained tumor tissue, but all failed to stain fat *in vivo*. They are, therefore, not included in the tables. As is shown in Table 1B, however, the corresponding 5-benzylamino compounds containing alkyl groups of more than 2 carbon atoms each on the 9-nitrogen did stain fat *in vivo*. While all of the dyes with propyl or butyl substituents in the 9-position stained fat, not all of them stained tumor tissue *in vivo*.

As was shown in earlier studies(1,6), the results obtained with Nile Blue staining of tumors in rats were inconsistent and unpredictable. Tumors became stained in only a few rats fed Nile Blue A or Nile Blue 2B. The staining depended to some extent upon the age of the rat and the size of the tumor. Four of the dyes tested in the present studies, however, gave satisfactory staining in mice and rats. The tumors were stained blue and the fat orange in rats and mice fed diets containing 5-benzylamino-9-dipropylamino, 5-benzylamino-9-dibutylamino, 5-(4-chlorobenzylamino)-9-dipropylamino or 5-(4-chlorobenzylamino)-9-dibutylaminobenzo[a]phenoxazine dyes.

5-naphthylamino- 9 -dialkylaminobenzo[a]-phenoxazine compounds behaved like phenyl derivatives in staining fat *in vivo*. A 6-membered nitrogen heterocyclic ring could also be substituted for the phenyl group without abolishing the *in vivo* fat-staining properties of the dyes.

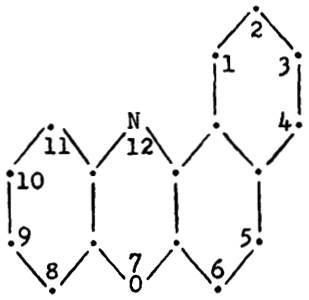
The results of experiments carried out in the present study showed that a number of the benzo[a]phenoxazine dyes were soluble in various types of fat, but only those containing the 5-phenylamino group or those having the hydrogens of the 9-amino group replaced by alkyl radicals containing more than 2 carbon atoms stained fat *in vivo*.

Discussion. Certain benzo[a]phenoxazine dyes stained fat *in vitro,* but not all of them were capable of staining fat *in vivo*. While the available evidence does not permit of a definite conclusion as to the mechanism of *in vivo* fat staining, it appears probable that it is related to the basicity of the dyes in question. The 5-phenylamino compounds stained fat *in vivo;* the corresponding derivatives having highly acidic substituents on the benzene ring did not.

7. Crossley, M. L., Dreisbach, P. F., Hofmann, C. M., Turner, R. J., and Parker, R. P., Abstracts of papers presented at Am. Chem. Soc. Meeting, Cleveland, April, 1951, p17L, to be published.

TABLE I. Staining Effects of Benzo [a] phenoxazine Dyes.† Substituted 5,9-diamino derivatives.

A. Derivatives containing a phenyl group on the 5-amino nitrogen (salt or base indicated)



	Staining		
	Tumor	Fat	Urine
5-phenylamino-9-dimethylamino, chloride	0	Salmon	Pale green
5-(2-methylphenylamino)-9-dimethylamino, nitrate	Pale green	,,	,, ,,
5-(3-methylphenylamino)- ,, chloride	,, ,,	Pink	,, ,,
5-(4-methylphenylamino)- ,, ,,	Pink	Peach	,, ,,
5-(2-chlorophenylamino)- ,, ,,	0	Pink	0
5-(3-chlorophenylamino)- ,, ,,	0	Salmon	0
5-(4-chlorophenylamino)- ,, ,,	0	Peach	0
5-(2-hydroxyphenylamino)- ,, ,,	0	0	Pale green
5-(3-hydroxyphenylamino)- ,, ,,	0	0	0
5-(3-hydroxy-4-carboxyphenylamino)-9-dimethylamino, chloride	0	0	0
5-(2-methoxyphenylamino)-9-dimethylamino, chloride	Orchid	Pink	Pale green
5-(4-N-2-pyrimidyl-sulfamylphenylamino)-9-dimethylamino, chloride	0	,,	,, ,,
5-phenylamino-9-ethylamino, chloride	Green blue	,,	Blue
5-phenylamino-9-diethylamino, chloride*	Blue	Red	Blue green
5-(2-methylphenylamino)-9-diethylamino, nitrate	Violet	,,	Green
5-(3-methylphenylamino)- ,, ,,	Pale violet	,,	Pale green
5-(4-methylphenylamino)- ,, chloride	Violet	,,	,, ,,
5-(4-ethylphenylamino)- ,, nitrate	,,	,,	,, ,,
5-(4-isopropylphenylamino)- ,, ,,	,,	,,	,, ,,
5-(4-t-amylphenylamino)- ,, ,,	0	Peach	,, ,,
5-(4-chlorophenylamino)- ,, chloride	? pink	Red	,, ,,
5-(2-hydroxyphenylamino)- ,, ,,	Pale green	0	Bright green
5-(3-hydroxyphenylamino)- ,, ,,	Violet	0	Pale green
5-(4-hydroxyphenylamino)- ,, ,,	0	0	,, ,,
5-(2-hydroxy-5-chlorophenylamino)-9-diethylamino, chloride	0	0	,, ,,
5-(4-carboxyphenylamino)- ,, ,,	0	0	0
5-(2-methyl-4-chloro-5-nitrophenylamino)-9-diethylamino, base	0	Pink	Yellow
5-(2-biphenylamino)-9-diethylamino, chloride	0	Peach	0
5-(4-N-2-pyrimidyl-sulfamylphenylamino)-9-diethylamino, chloride	0	Pale violet	0
5-(4-methylphenylamino)-9-isopropylamino, nitrate	Violet	Purple	0
5-phenylamino-9-dipropylamino, nitrate	,,	Red	Pale green
5-(2-methylphenylamino)-9-dipropylamino, nitrate	,,	,,	,, ,,
5-(3-methylphenylamino)- ,, ,,	,,	,,	,, ,,
5-(4-methylphenylamino)- ,, bromide	,,	Rose	0
5-(4-methylphenylamino)- ,, phosphate	,,	Scarlet	Pale green
5-(4-methylphenylamino)- ,, sulphate	,,	Rose	Blue
5-(4-ethylphenylamino)-9-dipropylamino, nitrate	Pale violet	Red	Pale green
5-(4-ethylphenylamino)- ,, sulphate	,, ,,	Rose	0
5-(4-isopropylphenylamino)- ,, nitrate	,, ,,	Red	0
5-(4-isopropylphenylamino)- ,, phosphate	Violet	,,	Pale green
5-(4-chlorophenylamino)- ,, nitrate	0	,,	,, ,,
5-(N-ethyl-N-phenylamino)- ,, ,,	? violet	,,	0
5-phenylamino-9-dibutylamino, nitrate	0	Pink	0
5-(4-methylphenylamino)-9-dibutylamino, nitrate	0	,,	0
5-(4-methylphenylamino)- ,, base	0	,,	0
5-phenylamino-9-diamylamino, base	Pink	,,	0

	Staining		
	Tumor	Fat	Urine
5-phenylamino-9-dihexylamino, base	0	0	0
5-(4-methylphenylamino)-9-methylpropylamino, base	0	Red	Yellow
5-phenylamino-9-ethylpropylamino, nitrate	Violet	"	Green
5-(4-methylphenylamino)-9-ethylpropylamino, nitrate	Pale violet	Scarlet	Pale green
5-phenylamino-9-butylpropylamino, nitrate	" "	Red	" "
5-(2-methylphenylamino)-9-butylpropylamino, nitrate	" "	"	Yellow
5-(4-chlorophenylamino)- " "	Pink	"	Pale green
5-phenylamino-9-(4-morpholinyl), chloride	0	Orange	0
5-(4-methylphenylamino)-9-(4-morpholinyl), chloride	Green blue	"	0
5-(4-methylphenylamino)-9-diethylamino-10-methyl, chloride	Violet	Russet	Pale green
5-phenylamino " -11-methyl, nitrate	0	Pale pink	Orchid
5-(2-methylphenylamino)- " " "	0	Pink	0
5-(4-methylphenylamino)- " " "	Pale blue	Red	Pale green
5-(4-methylphenylamino)- " " base	Blue	Rose	" "
5-(4-chlorophenylamino)- " " nitrate	0	Pink	Pale violet
5-phenylamino-9-ethylamino-10 (or 8)-methyl, nitrate	0	Salmon	Pale green
5-(4-methylphenylamino)-9-ethylamino-10 (or 8)-methyl, nitrate	?	Red	" "
B. Derivatives containing a group on the 5-amino nitrogen, other than a phenyl group (salt or base indicated)			
5-(1-naphthylamino)-9-diethylamino, chloride	? blue	Red	0
5-(2-naphthylamino)- " "	0	Pink	0
5-benzylamino-9-dipropylamino, chloride	Blue	Orange	Pale blue
5-(4-methylbenzylamino)-9-dipropylamino, chloride	Pale blue	Pink	Blue
5-(4-chlorobenzylamino)- " "	Blue	Salmon	"
5-benzylamino-9-dibutylamino, chloride	Deep blue	"	Pale blue
5-(4-methylbenzylamino)-9-dibutylamino, chloride	Blue	Orange	" "
5-(4-chlorobenzylamino)- " "	"	Salmon	" "
5-benzylamino-9-diamylamino, chloride	"	"	? green
5-(4-methylbenzylamino)-9-diamylamino, nitrate	"	"	Pale green
5-(4-chlorobenzylamino)- " chloride	Pale blue	Peach	" "
5-benzylamino-9-dihexylamino, chloride	Blue	Russet	" "
5-(4-methylbenzylamino)-9-dihexylamino, nitrate	"	Salmon	" "
5-(4-chlorobenzylamino)- " chloride	Pale blue	"	" "
5-(2-pyridylamino)-9-diethylamino, chloride	Pale green	Rose	" "
5-(2-pyridylamino)-9-dipropylamino, nitrate	0	"	Green
5-(2-pyrazinylamino)- " "	Violet	Purple	0
5-(2-pyridylamino)-9-dibutylamino, nitrate	"	Rose	Pale green

* N-Phenyl Nile Blue A Chloride.

† The expressions used to designate color in the tables are based on the visual observation of the investigator.

Microscopical examination of the stained fatty tissue showed that the color was confined to the fat globule, the surrounding cytoplasm remaining uncolored. Fat globules in macrophages and supporting tissue were stained the same color as those in the fatty tissue. As may be seen in the tables, the color exhibited by the fatty tissue in the treated animals depended upon the particular dye ingested. The majority of the dyes stained fatty tissue tones or orange and red, although a few stained the fat tones of pink and purple. The color which appeared in the tumors of the treated mice also varied depending upon the dye ingested; the color of the tumors was not the same as that of the fat in the same animal. In general tumor tissue became stained blue or tones of blue mixed with red or with yellow.

Summary. (1) 5-phenylamino-9-dialkylaminobenzo[a]phenoxazine dyes stained fat *in vivo* when administered to mice in their food. (2) The fat staining properties of the dyes were not materially altered by substituents on the phenyl nucleus other than those of highly acidic nature, or by substitutions of the naphthyl group and certain nitrogen heterocyclic rings for the phenyl group. The 5-phenylamino-9-morpholinyl derivatives also stained fat *in vivo*. (3) 5-benzylamino-9-dimethylamino- and diethylaminobenzo[a]phenoxazine dyes did not stain fat *in vivo*, al-

though they did stain tumor tissue. The corresponding dyes in which the alkyl group on the 9-nitrogen contained more than 2 carbon atoms stained fat *in vivo*. These dyes also stained tumor tissue *in vivo*.

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Studies of Proteolytic Enzyme Systems in Patients with Emotional Disorders.* (19087)

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(Introduced by C. M. Carpenter.)

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Studies of protein metabolism have revealed well-defined deviations in the concentrations of certain protein-splitting enzymes in the sera of patients with cancer(1-3). This report concerns the existence of analogous enzymatic disturbances in the presence of emotional disorders. The enzymes in question are accompanied in the blood by excesses of their inhibitors. Measurements of the latter permit valid deductions referable to the particular enzyme systems. Chymotrypsin-inhibitor is one element of an enzyme system which correlates with the rate of protein destruction. For example, abnormal concentrations have been noted in the presence of cancer, infections, physical trauma and wasting diseases. The relief from anxiety afforded by prefrontal lobotomy(4) for the alleviation of intractable pain of cancer, is often followed by significant reductions in abnormally increased concentration of chymotrypsin-inhibitor. Electric convulsive therapy provides comparable results. Therefore it becomes important to know the effect of emotional status alone upon the titer of chymotrypsin-inhibitor. Under certain circumstances its concentration in the serum correlates with the intensity of emotional reaction. In a series of 102 patients, the degree of anxiety experienced by each was estimated clinically (Fig. 1.) A graded scale of 5 divisions was employed: absence of anxiety and panic represented the extreme categories; slight, moderate and severe anxiety were defined in the intermediate groups. Only schizophrenic psychotics, conversion hysterics and patients with acute anxiety states were included. A direct correlation between the degree of anxiety and elevations of chymotrypsin-inhibitor concentrations was found to exist in the last group alone. Patients with conversion hysteria exhibited no such relationships, and furthermore seldom demonstrated more than slight elevations in titer. On the other hand, schizophrenic psychotics showed highly variable enzymatic changes, some of which were directly related to the level of anxiety, while the majority were not. But, in this group high titers *were* obtained.

It then became imperative to investigate the possibility that the changes in protein metabolism might be associated with variations in adrenal function. The demonstration that the urinary excretion of the proteolytic enzyme, uropepsin, is an appropriate measure of adrenal activity, the titer directly paralleling adrenal function(5), provided a satis-

* Reviewed in the Veterans Administration and published with the approval of the chief medical director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinions or policy of the Veterans Administration.

1. West, P. M., and Hilliard, J., *Ann. West. Med. and Surg.*, 1949, v3, 227.

2. West, P. M., and Hilliard, J., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 169.

3. West, P. M., Rapaport, S. I., and Tempereau, C. E., *Cancer*, 1951, v4, 177.

4. French, J. D., Personal communications.

5. Spiro, H. M., Reifenstein, R. W., and Gray, S. J., *J. Lab. and Clin. Med.*, 1950, v35, 899.