TABLE II. Calculation of Posthemorrhage Plasma Volume from Changes in Protein Concentration.

Dog No. and sex	Date of exp.	% BV remaining	PV_1 (ml)	$(P.P.)_1$ (g %)	Total $(P.P.)_1$ (g)	P.P. out (g)	Total $(P.P.)_2$ (g)	$(P.P.)_2$ (g %)	PV_{2pp} (ml)	$PV_{2T-1824}$ (ml)	% dev.*
14 ♀	4/24/59	80.8	391	6.16	24.09	5.01	19.08	5.66	337	340	− .9
13 ♂	4/22/59	77.4	545	6.31	34.39	8.22	26.17	5.61	466	441	+5.7
10 ♀	4/17/59	77.1	556	6.36	35.36	9.76	25.60	5.20	492	467	+5.4
11 ♀	8/15/58	75.7	377	5.35	20.17	5.83	14.34	4.29	334	318	+5.0
8 ♂	3/24/59	75.4	447	5.66	25.30	6.56	18.74	5.30	354	372	−4.8
9 ♂	8/25/58	74.9	604	6.42	38.78	9.67	29.11	5.91	493	472	+4.4
12 ♂	8/27/58	74.1	682	6.62	45.15	13.99	31.16	5.81	536	542	−1.1
9 ♂	7/31/58	73.9	583	6.56	38.24	9.80	28.44	5.96	477	448	+6.5
17 ♀	8/ 5/59	73.6	363	5.15	18.69	5.23	13.46	4.60	293	286	+2.2
10 ♀	8/13/58	73.5	547	5.96	32.60	10.68	21.92	4.95	443	450	−1.6
18 ♀	8/13/59	73.4	538	5.30	28.51	7.98	20.53	4.60	446	420	+6.2
15 ♀	5/15/59	73.0	608	6.26	38.06	13.37	24.69	5.25	470	505	−6.8
7 ♂	8/ 4/58	72.8	647	6.31	40.83	11.89	28.94	5.45	531	505	+5.1
10 ♀	8/29/58	68.6	636	5.81	36.95	11.72	25.23	5.20	485	454	+6.8
11 ♀	11/10/58	66.2	386	5.76	22.23	8.57	13.66	5.05	270	284	−4.9

* % deviation $= \frac{PV_{2pp} - PV_{2T-1824}}{PV_{2T-1824}} \times 100.$

tomized-splenectomized dogs, it is better estimated from changes in plasma protein concentration than from cell volume and hematocrit.

Summary. In 6 out of 25 experiments on sympathectomized - splenectomized dogs, the posthemorrhage F_{cells} factor decreases to below the control range. The constancy of F_{cells} factor following hemorrhage in splenectomized dogs is partially due to sympathetic activity.

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Determination of N,N′-Diphenyl-p-Phenylenediamine in Animal Tissues.* (25971)

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Interest in the physiological activity of N,N′-diphenyl-p-phenylenediamine (DPPD) stems from its ability to substitute for Vit. E in prevention and cure of tocopherol deficiency diseases, including resorption gestation and muscular dystrophy(1,2). That this effect is not due to conservation of tocopherols in the tissues is evident from the fact that rate of decline of Vit. E in the liver of animals fed a diet deficient in the vitamin is not affected by DPPD administration, and that the antioxidant has curative as well as preventive activity(2). Following administration of d-α-tocopherol-5-methyl-C^{14} to rats, most of the radioactivity in the liver is present in the mitochondria as α-tocopherol, the remaining

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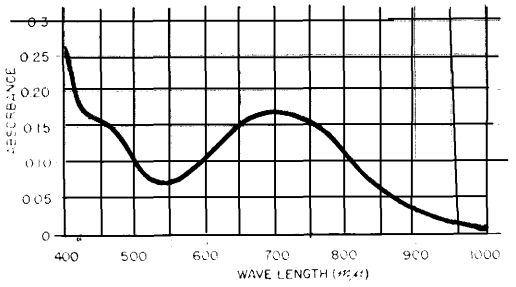

FIG. 1. Absorbance in ethanol of the product of oxidation of DPPD with ferric chloride.

counts being located primarily in the microsomes as unidentified metabolites(3). In view of the apparent nutritional interchangeability of Vit. E and DPPD, the present study was designed to determine the extent to which DPPD accumulates in the tissues and to compare its intracellular distribution with that of α-tocopherol. A new procedure was developed for estimation of DPPD in tissues, based on spectrophotometric measurement of the bluish green color produced by mild oxidation with $FeCl_3$. This method affords greater sensitivity than the semiquantitative procedure used by Pudelkiewicz and coworkers(4) for estimation of DPPD in avian tissues.

Chemical method. Solutions of DPPD in organic solvents are slowly oxidized by atmospheric oxygen to produce a color which ranges, depending upon concentration, from a light yellowish brown to a deep reddish brown. The oxidation is strongly catalyzed by heat and light, and cannot be reversed by sodium bisulfite. Oxidation with H_2O_2 or $FeCl_3$ leads to formation of a bluish green intermediate which can be bleached with sodium bisulfite or which, upon standing, further oxidizes to form the brown product. When the reaction is carried out in ether or acetone the existence of the intermediate is transitory but in ethanol, at room temperature and in dim light, the green color is stable for at least 15 minutes. The absorption curve for this compound, determined using the Beckman DU spectrophotometer, is shown in Fig. 1. A standard curve, based on absorbance at 700 mμ ($E^{1\%}_{1\text{ cm}} = 224$), followed the Beer-Lambert Law and permitted estimation of DPPD over a range of from 6 γ to at least 180 γ/3 ml sample. Sample measurements were made in the above instrument after addition of 1.0 ml of a solution of ethanolic $FeCl_3$ (0.1 g/100 ml) to the sample (or a suitable aliquot) in 2 ml absolute ethanol. *Preparation of samples.* Acetone rather than ethanol was used for extraction of animal tissues because of the greater solubility of DPPD in acetone and the greater ease with which lipids could be removed subsequently by crystallization. Liver samples (about 10 g) were ground with anhydrous Na_2SO_4 until dry and extracted overnight with acetone in darkness at room temperature on a mechanical shaker. Skeletal muscle samples were ground with a mixture of Na_2SO_4 and acid-washed sand. Saponification of fat samples with alcoholic KOH in presence of DPPD resulted in partial oxidation of DPPD, even in presence of pyrogallol and in darkness(5). Consequently the lipids were removed by low-temperature crystallization. After filtering the acetone extracts through No. 1 Whatman paper and washing, volume was reduced to 30-40 ml by vacuum distillation at 40°C under N_2 and the lipids were crystallized by placing the extracts in a dry ice-acetone bath at –70°C for 30 minutes. The lipids were removed by gentle vacuum filtration through No. 1 Whatman paper using a funnel surrounded by a dry ice-acetone jacket. The residue was washed 5 times with 10 ml precooled acetone and the filtrate was evaporated *in vacuo* under N_2 at 40°C, the residue being dissolved in a suitable volume of ethanol. The reaction mixture consisted of 2 ml of this ethanolic solution to which was added 1 ml of $FeCl_3$ solution. Optical density of the sample was read against a reagent blank at 700 mμ. Some samples contained small amounts of yellow pigments which necessitated the use of a sample blank. Known quantities of DPPD added to lard were recovered quantitatively by the above procedure. *Animal experiment.* To determine the degree to which DPPD is accumulated in the liver and skeletal muscle of rats fed different levels of this compound, 3 groups of 3 rats each, weighing about 350 g, were maintained for 7 days on a commercial stock diet containing .005%, .05% or .5% DPPD. After sacrifice, livers and samples of thigh muscle were removed and prepared according to the procedure outlined. *Cell fractionation experiment.* Subcellular distribution of DPPD was determined

TABLE I. Concentration of DPPD in Liver and Skeletal Muscle of Rats Receiving Different Dietary Levels (3 Rats/Group).

Conc. in diet, %	Conc. in tissues, γ/g fresh wt	
	Liver	Skeletal muscle
.005	4.1- 4.5	2.4-2.5
.05	8.9- 9.3	4.3-4.9
.5	11.0-13.9	7.7-8.0

using livers from 3 rats which had been fed a commercial stock diet containing .05% DPPD for 7 days. Livers were removed under ether anesthesia and homogenized in 9 volumes of 0.25 M sucrose using a smooth-walled glass homogenizer and serrated Teflon pestle attached to a drill press. Cell fractions were prepared in a Servall refrigerated centrifuge essentially according to the procedure of Schneider(6) except that the microsomal fraction was prepared by centrifuging at 25,000 g for 90 minutes. The mitochondrial and microsomal fractions were dried and extracted as described for liver whereas the supernatant was lyophilized before extraction. The extracted residues were analyzed for nitrogen by the microKjeldahl procedure(7).

Results. The data shown in Table I indicate concentrations of DPPD in liver and muscle which result from feeding 3 different levels in the diet. An increase of 100-fold in dietary intake induced only a 3-fold increase in tissue concentration. The inefficiency with which this compound is absorbed from the intestine(8) probably accounts for its failure to accumulate in greater amounts in the tissues. Nevertheless, intakes of .005% or lower are best suited to maintaining reproductivity on a tocopherol-deficient regimen owing to the high order of chronic toxicity exhibited by DPPD for the pregnant female rat(1).

The intracellular distribution of DPPD, based on total recovery in the 3 fractions shown, is given in Table II. Over half was recovered in the supernatant, about one-quarter in the microsomal fraction and a small portion in the mitochondria. It is of interest to compare these results with those for intracellular distribution of lipids. With due allowances for differences in the method used for preparation of cell fractions, the data of Clement and coworkers(9) depict a generally similar distribution pattern for total fatty acid esters as well as for various lipid fractions. These results indicate that distribution of DPPD may be determined largely by its solubility in intracellular lipid.

It is also of interest to compare these findings with those for distribution of the natural antioxidant α-tocopherol (Table II). After administration of C^{14}-labelled d-α-tocopherol to rats, about 60% of the radioactivity in the liver is located in the mitochondria, about half as much being present in the microsomes and only a small fraction in the supernatant. Moreover, the counts recoverable from mitochondria are due primarily to free α-tocopherol whereas the labelled compounds in the other fractions are predominantly metabolites(3). Thus, in contrast to DPPD, the distribution of which appears to reflect distribution of total lipid, α-tocopherol apparently is concentrated in the mitochondria. The implications of this observation in regard to the demonstrated ability of DPPD to substitute for Vit. E *in vivo*, and for the metabolic role of α-tocopherol, are not clear.

Summary. In connection with a study of replacement of Vit. E *in vivo* by certain synthetic antioxidants, liver uptake and intracellular distribution of the Vit. E-active compound N,N′ - diphenyl - p - phenylenediamine (DPPD), following its administration to rats, has been determined by a method based on spectrophotometric measurement of the color developed by oxidation with ferric chloride in ethanol. Over half of the DPPD recovered was present in the soluble fraction of liver

TABLE II. Subcellular Distribution of DPPD in Rat Liver.

	Mitochondrial fraction		Microsomal fraction		Supernatant	
	Range	Avg	Range	Avg	Range	Avg
DPPD content (basis 3 livers), γ	17.6-21.7	19.3	31.5-45.1	36.8	61.4-84.3	74.3
DPPD distribution, %	13.8-15.6	14.8	23.5-31.3	28.2	53.6-62.7	57.0
C^{14} distribution after C^{14}-d-α-tocopherol administration (3), %		60.2		32.5		7.3

cells, one-quarter was associated with microsomes, and a small fraction recovered from mitochondria. This distribution pattern is similar to that reported for total lipids, and indicates that the intracellular location of DPPD may be passively determined by its lipid solubility. Alpha-tocopherol, on the other hand, was found predominantly in mitochondria.

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Water Diuresis Stop Flow Analysis in the Dog.* (25972)

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When the technic of stop flow analysis was described(2,3) it appeared to offer a means to evaluate more fully the mechanism for control of urea excretion by the kidney. Our data from urea stop flow analyses performed during osmotic diuresis were difficult to interpret. Accordingly, we modified the method using water diuresis. This did not aid in interpretation of the urea data but did provide interesting findings concerning stop flow analysis. During water diuresis stop flow analyses the sodium U/P minimum appeared near the peak of the para-aminohippurate (PAH) curve and potassium and osmolal U/P maxima were distal to the sodium U/P minimum.

Method. The following procedure was found successful in producing water diuresis with rates of urine flow of 6 to 11 ml/min./kidney in the pentobarbital anesthetized dog. Alcohol was administered to the dog in drinking water or by intravenous route. The dog was then anesthetized with pentobarbital. Four hundred ml water were administered by gavage. An infusion of 0.4% NaCl and 2% glucose in water was administered by intravenous route with 10 g alcohol in each liter of infusion. The left ureter was cannulated avoiding the use of cautery. After diuresis had become brisk PAH and creatinine were added to the infusion. When rate of urine flow had become stable and greater than 6 ml/min./kidney the control clearance collections were begun.

Three 10 minute control clearance urine collections were obtained before, and 2 after, the stop flow procedure. The ureteral cannula was occluded for 8 minutes with inulin given by intravenous route 2 minutes before release of the occlusion. Upon release of the occlusion rapid serial collections were obtained from the ureteral cannula for a period of 3 minutes. Heparinized arterial blood samples were obtained at the mid-point of each control clearance urine collection and at time of occlusion, release of occlusion, and termination of the stop flow serial collections.

Osmolalities were performed with a microthermister using the Fiske osmometer. Determinations were made on urine and plasma

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