able effect on leukocyte alkaline phosphatase activity and zinc or magnesium have no activating effect. Obviously, there is a species difference either in the leukocyte or in the kind of phosphatase present. By histochemical studies we have been able to show a marked increase in the leukocyte alkaline phosphatase of the rabbit following injection of vaccines, pyrogens, etc., but the enzyme type has not as yet been characterized.

Freiman(10) has carried out rather comprehensive histochemical studies of inhibition of alkaline phosphatase activity of various tissues by EDTA and concluded that a number of alkaline phosphatases exist. Our findings indicate that even a brief incubation of human leukocytes with EDTA results in a decrease in alkaline phosphatase activity. EDTA currently enjoys much popularity as an anticoagulant for blood and is widely used in tissue decalcification. Obviously the chelating properties of EDTA for ions other than calcium must be kept in mind in any investigation of alkaline phosphatase activity in blood or tissues.

Summary. EDTA inhibits alkaline phosphatase activity of the human leukocyte. The rabbit leukocyte is, however, not so affected. There seem to be 2 alkaline phosphatases in the human leukocytes: one requiring magnesium and the other zinc for its activation.

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Received February 5, 1957. P.S.E.B.M., 1957, v95.

Spleen DPNH Cytochrome *c* Reductase Activity In X-Irradiated Rats.* (23111)

HERBERT J. EICHEL
Division of Biological Chemistry, Hahnemann Medical College, Philadelphia, Pa.

With either α-ketoglutarate or succinate as substrate, several laboratories(1-3) have reported a decrease in oxidative phosphorylation by mitochondria and homogenates prepared from spleens of rats and mice exposed to lethal doses of X-irradiation. Oxygen consumption was also found to be diminished in the presence of either α-ketoglutarate(2,3) or malate(4). Since phosphorylation is coupled to DPNH oxidation in the sequence DPNH → cytochrome *c*, it was considered of interest to determine whether interference with DPNH cytochrome *c* reductase activity in the spleens of X-rayed animals was involved in the lowered oxidation rates or linked in some way to the diminished P:O ratios. Another area on which some light might be shed by the study of DPNH cytochrome *c* reductase activity in irradiated animals is in the relation of cell structure to function. The presence of a high concentration of this enzyme in liver(5) and spleen† microsomes is somewhat of an oddity, since most of the known linked respiratory systems are associated with the mitochondria. Loss of microsomal DPNH cytochrome *c* reductase to the exclusion of the mitochondrial enzyme would appear to cast

* Supported by contract with Division of Biology and Medicine, U. S. Atomic Energy Commission.

The following abbreviation is employed: DPNH = reduced diphosphopyridine nucleotide.

† Eichel, H. J., *J. Biophys. and Biochem. Cytology,* 1957, v3, no. 3, in press.

the latter in a more significant physiological role.

The effect of 700 r of whole-body X-irradiation on the DPNH cytochrome *c* reductase activity of rat spleen homogenates, and subcellular fractions isolated from them, is described here.

Methods. A. Irradiation procedure and experimental design. Two series of experiments were performed with male rats obtained from the Wistar Institute. In the first series at the time of irradiation, the animals weighed from 110 to 150 g, and in the second, from 130 to 155 g. The rats were given, in a single exposure, a total dose of 700 r of 220 KV X-rays filtered through 0.25 mm Cu and 1 mm Al at an average rate of 87.1 r/minute and a target distance of 50 cm. The animals were irradiated in groups of 6 in metabolism cages. Food was withheld from exposed and control rats for the first 20 to 22 hours after irradiation. Purina dog chow checkers were then fed to both groups, but to correct for any changes in enzyme activity that might be caused by the decreased food intake of the irradiated rats, the control animals were carefully pair-fed against the exposed. Water was provided *ad libitum*. In the first series, rats were killed by a blow on the head, and in the second, by ether anesthesia. Six control and 6 experimental rats were sacrificed on the first and fourth days after irradiation in the first experiment. In the second experiment, 6 rats from each group were sacrificed on the first, second, and fourth days after exposure. Owing to the relatively long time required to complete each fractionation, it was deemed advisable to pool the spleens of 3 animals for a single fractionation. Hence, on all but 1 day, 4 homogenates and 4 complete fractionations were made, 2 each with spleens grouped from 3 X-rayed rats and 2 each with organs from 3 control animals. On day 4 of the first experiment, only homogenates were prepared. The first, second, and fourth days after irradiation actually correspond to 13, 37, and 85 hours post-irradiation. *B. Preparation and fractionation of spleen homogenates.* After excision, spleens were washed free of adhering blood and homogenized in cold 0.25 M sucrose with a Ten Broeck glass homogenizer. Each homogenate (3 spleens) was diluted to 10 ml with sucrose, 2 ml were removed for nitrogen analysis, and the remainder was fractionated, with some changes, according to the method of Hogeboom *et al.*(6) as modified by Schneider and Hogeboom(7). All operations were carried out at 0–4° C. The nuclear fraction was collected by centrifugation at 600 X g for 10 minutes, the mitochondria by centrifugation at 5,000 X g for 15 minutes, and the microsomes and "fluffy layer" by centrifugation at 110,000 X g for 60 minutes. The nuclear and mitochondrial fractions were each washed once with sucrose. Four such fractionation procedures, performed simultaneously, required about 6 hours to complete. A study of the intracellular localization of DPNH cytochrome *c* reductase and cytochrome *c* oxidase in fractions derived from normal rat spleen homogenates has been reported. A detailed account of this and related studies will be published elsewhere.† The recovery of most of the deoxypentosenucleic acid in the 600 X g fraction (nuclear) and 45% of the ribonucleic acid in the 110,000 X g fraction (microsomes), the high specific and total activities of succinic dehydrogenase and cytochrome *c* oxidase in the 5,000 X g fraction (mitochondria), and the low specific and total activities of these enzymes in the 110,000 X g fraction provide strong chemical and enzymatic evidence that the isolated fractions studied here are analogous to those which have been separated from liver homogenates at similar centrifugal forces. The distribution pattern of DPNH cytochrome *c* reductase activity in the particulate fractions of spleen homogenates is similar to that observed by Hogeboom for liver(5) except that, per mg of nitrogen, the enzyme is concentrated to a much greater degree in spleen mitochondria. This is probably a reflection of the fact that the mitochondrial fraction represents only about 5-8% of the total nitrogen content of spleen homogenates. *C. Enzyme assay, materials.* DPNH cytochrome *c* reductase activity was determined spectrophotometrically (Beckman DU) at 25°C essentially according to the method of Potter and Albaum(8). The following components were added to each cuvette in the

order given: 0.025 to 0.2 ml of a suitable dilution of homogenate or fraction, 0.2 ml of 0.03 M KCN, 0.3 ml of 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4, water to make a volume of 3 ml, 1.43 mg of cytochrome *c* in 0.25 to 0.75 ml of water, and 0.3 to 0.4 micromole of DPNH in 0.7 ml of 0.1 M KH_2PO_4–K_2HPO_4, pH 7.4. The omission of nicotinamide did not affect reductase activity. Zero time was taken as time of mixing (by inversion) of the covered cuvette immediately after addition of DPNH. The first reading, at 550 mμ, was the time required for the instrument to reach a predetermined density value. Subsequent readings were made at densities 0.010 or 0.020 above the preceding ones until 6 to 10 readings were taken, or, in the case of the supernatant fraction, 2 to 3 minutes had elapsed. Activity of each fraction (with exception of the supernatant) was reasonably proportional to the amount of tissue present. The frequent absence of proportionality between activity of the supernatant fraction and the volume tested made accurate calculations of the absolute activity of this fraction difficult. The reason for this phenomenon is not known. The specific activity of homogenates and all fractions was calculated with the value 1.96 $\times 10^4$ sq cm/mole as the difference between molecular extinction coefficients of oxidized and reduced cytochrome *c*. The reaction rates were zero order with respect to cytochrome *c* concentration, and specific activity was expressed as *micromoles of cytochrome c reduced/minute/mg of nitrogen* (of each fraction added to the cuvette). Total activity (micromoles of cytochrome *c* reduced/minute) was obtained by multiplying the specific activity of each homogenate or fraction by its respective nitrogen content. Nitrogen was determined by a micro-Kjeldahl procedure. The DPNH was prepared chemically with sodium hydrosulfite(9) from DPN (Sigma Chemical Co.) which was 80% pure by alcohol dehydrogenase assay(10). Beef heart cytochrome *c* of about 70% purity, based on extinction coefficients of oxidized and reduced cytochrome *c*, was obtained from Sigma.

Results. The distribution of nitrogen in the subcellular fractions isolated from spleen homogenates of control and X-rayed rats is presented in Table I. Due to splenic atrophy following X-irradiation, it can be seen that in each experiment on the first day post-irradiation, spleens of X-rayed animals contained 25 to 35% less nitrogen than did those of controls. In Exp. No. 2, day 2, the nitrogen content of control spleens was essentially unchanged while that of the X-rayed organs diminished to an even greater degree. By day 4, in each experiment, the nitrogen content of X-rayed spleens was still further reduced while that of the control spleens had fallen by 40% as compared to day 1 in Exp. No. 1 and by 60% as compared to days 1 and 2 in Exp. No. 2. In each experiment, data for average body weights of control animals for day 4 suggest that the decreased nitrogen content (and wet weight) of the spleens was probably a result of acute inanition imposed by pair-feeding. In Exp. No. 2, the average body weights (g) of the 6 X-rayed rats studied on each of days 1, 2, and 4 were 137, 127, and 110, respectively, and of the 6 control rats, 144, 135, and 110, respectively. In Exp. No. 1, body weights of X-rayed animals averaged 139 and 127 g on days 1 and 4, respectively, while those of the control rats averaged 124 and 116 g, respectively.

The data of Table I also show that the % recovery of homogenate nitrogen in spleen mitochondria from irradiated rats was twice as high as from control animals. This was also observed by Maxwell and Ashwell in spleen mitochondria obtained from X-irradiated mice(1). They concluded that some of the tissue debris produced by irradiation sediments as inert nitrogenous material with the mitochondria. The % recovery of nitrogen in spleen microsomes and supernatant from irradiated rats was also slightly higher than from control animals. On the other hand, more nitrogen was recovered in the nuclear fractions of spleens from control rats than from exposed animals. It is worthwhile noting that the % recoveries of nitrogen in mitochondrial fractions of control spleens listed in Table I were less than those (8%) found in earlier experiments.† The explanation for this difference is not known, although in the earlier work, the rats were fed *ad libi-*

TABLE I. Distribution of Nitrogen in Subcellular Fractions Isolated from Spleens of Control and Irradiated Rats. Each value represents avg of 3 pooled spleens.

			Total nitrogen (mg)				Recovery (%)			
			Irradiated		Control		Irradiated		Control	
Exp. No.	Day	Fraction	1	2	1	2	1	2	1	2
1	1	H*	25.6	28.3	36.2	34.4	100	100	100	100
		N	11.7	12.8	20.1	19.5	45.7	45.3	55.5	56.5
		M	2.5	2.8	1.8	1.7	9.7	10.0	4.9	4.9
		P	2.7	3.2	3.5	3.5	10.6	11.3	9.6	10.2
		S	7.7	8.4	9.3	8.8	30.2	29.8	25.6	25.5
		Recovery	24.6	27.2	34.7	33.5	96.2	96.4	95.6	97.1
	4	H	12.5	12.3	23.3	16.7				
2	1	H	24.8	22.4	36.1	36.7	100	100	100	100
		N	11.9	9.8	21.2	21.9	48.0	43.8	58.8	59.6
		M	2.0	1.9	1.7	1.7	8.2	8.5	4.7	4.6
		P	3.1	2.8	3.5	3.5	12.5	12.3	9.6	9.5
		S	6.8	6.7	8.6	8.6	27.6	29.8	23.7	23.3
		Recovery	23.8	21.2	35.0	35.7	96.3	94.4	96.8	97.0
	2	H	16.1	17.8	41.0	38.8	100	100	100	100
		N	6.3	7.8	23.9	22.9	39.0	44.0	58.4	60.0
		M	1.5	1.5	1.8	1.8	9.3	8.2	4.4	4.6
		P	2.2	2.1	3.9	4.0	13.4	12.0	9.6	10.2
		S	4.9	5.5	10.3	10.5	30.5	31.0	25.1	27.1
		Recovery	14.8	16.9	39.9	39.1	92.2	95.2	97.5	101.9
	4	H	9.2	10.1	16.0	14.6	100	100	100	100
		N	3.8	3.9	7.8	7.1	41.3	38.3	48.9	48.2
		M	1.1	1.0	1.1	1.0	11.4	10.2	7.1	6.6
		P	1.3	1.3	1.9	1.7	13.8	12.9	11.6	11.8
		S	2.5	3.1	3.8	4.0	27.6	30.4	24.0	27.1
		Recovery	8.7	9.3	14.7	13.7	94.1	91.8	91.6	93.7

* H = homogenate, N = nuclei, M = mitochondria, P = microsomes, S = supernatant.

tum.

Distribution of DPNH cytochrome *c* reductase activity in fractions obtained from spleen homogenates of control and irradiated rats is presented in Table II. On the first day following irradiation, specific activities of the homogenates of X-rayed spleens were increased by an average of 43 and 42% in Exp. No. 1 and 2, respectively. On day 2 of Exp. No. 2, the activities of these homogenates were again 42% higher than the control levels. On day 4 of Exp. No. 2, the activities of the 2 control homogenates had risen by 36% over the average control value for day 1. Therefore, while the activities of X-rayed homogenates were still elevated, their average figure for day 4 was only 13% greater than the average control activity for that day. The data of Table II show a similar relationship between the homogenate activities of days 1 and 4 in Exp. No. 1. It is likely that the increased activities of control homogenates on day 4 were a reflection of the loss of general spleen nitrogen due to the severely restricted food intake of the control rats. If non-cytochrome reductase nitrogen were lost more rapidly than reductase nitrogen, the net result would be an increased concentration of enzyme with respect to nitrogen. Activities of control homogenates and their nitrogen content on day 2, Exp. No. 2 are in agreement with this idea. The data in Table I show that on day 2 nitrogen content (essentially proportional to wet weight) of control spleens was unchanged when compared to nitrogen content of control spleens of day 1, and in Table II, little change in enzyme activities of control homogenates of day 2 is noted as compared to values of day 1. Thus, under the experimental conditions described, the interval between days 2 and 4 was critical in effecting a great reduction in nitrogen content of spleens of control rats. The view is favored here that the early increased specific

TABLE II. Distribution of DPNH Cytochrome *c* Reductase Activity in Subcellular Fractions Isolated from Spleens of Control and Irradiated Rats. Each value represents the avg of 3 pooled spleens.

			Specific activity*			
			Irradiated		Control	
Exp. No.	Day	Fraction	1	2	1	2
1	1	H†	.317	.320	.232	.214
		N	.100	.111	.104	.123
		M	.838	.784	1.149	1.230
		P	1.118	.985	.885	.909
		S	.094	.077	.076	.080
	4	H	.392	.402	.340	.366
2	1	H	.364	.419	.248	.304
		N	.110	.125	.141	.139
		M	1.197	1.138	1.638	1.612
		P	.923	.989	.840	.916
		S	.170	.180	.158	.179
	2	H	.435	.388	.288	.292
		N	.145	.116	.133	.118
		M	1.157	1.053	1.691	1.475
		P	1.034	.959	.735	.757
		S	.200	.190	.160	.158
	4	H	.456	.418	.401	.371
		N	.180	.157	.185	.193
		M	1.126	1.056	1.528	1.736
		P	.992	.902	1.123	1.080
		S	.200	.190	.120	.100

* Micromoles of cytochrome *c* reduced/min./mg of nitrogen.

† H = homogenate, N = nuclei, M = mitochondria, P = microsomes, S = supernatant.

activities of the homogenates prepared from spleens of irradiated animals were also due to loss of non-enzyme nitrogen rather than to a true activation of the enzyme. In the case of irradiated rats, the decrease in spleen nitrogen content (35% less than the controls) was apparent by day 1 and was accompanied by a 42% increase in specific activity of DPNH cytochrome *c* reductase. However, it should be noted that between days 1 and 4, a further marked drop in nitrogen content of X-rayed spleens occurred (60% less than the X-rayed levels for day 1), but reductase activity increased by only an additional 12%. This observation might be interpreted to mean that during this interval a considerable amount of enzyme was lost as well as general spleen nitrogen.

Specific activities of the nuclear, microsomal, and supernatant fractions after irradiation showed no striking changes as compared to control values (Table II). However, specific activities of the mitochondrial fractions from spleens of the irradiated animals were decreased by about 30% as compared to control activities. This decrease was probably due, in part, to the fact that the % of whole homogenate nitrogen recovered in mitochondria after irradiation was twice that found in mitochondria derived from control spleens.

In Exp. No. 2, total DPNH cytochrome *c* reductase activity (micromoles of cytochrome *c* reduced/minute) of spleen homogenates from control rats averaged 10.1, 11.6, and 5.9, and that of homogenates from the X-rayed rats averaged 9.2, 7.0, and 4.2 on the first, second, and fourth days after irradiation, respectively. Compared to control levels, this amounted to a loss by the X-rayed spleens of 9, 40, and 30% of the total reductase activity on the 3 days. In Exp. No. 1, by the fourth day, total enzyme activity of X-rayed spleens also averaged 30% less than that of controls, although on the first day it was 9% higher than the control values. On each day, of the total enzyme activity of the spleen homogenates from either the control or irradiated animals, 25 to 30% was recovered in the mitochondria and 30 to 37% in the microsomes. The total reductase activity recovered in the nuclear fractions of X-rayed spleens on each day was only about half that found in the nuclear fractions of control spleens. This might have been due to (1) the somewhat greater nitrogen recovery in the nuclear fractions from control spleen homogenates and (2) the fact that an average of only 72% of the total activity of the original homogenates was recovered among all fractions prepared from irradiated spleens while 88% of the total activity was recovered among the 4 fractions obtained from the control spleens. In a previous study, the % recovery of total DPNH cytochrome *c* reductase activity from normal rat spleen homogenates ranged from 90 to 100 (avg = 103) for 10 separate fractionations.†

The unimpaired DPNH cytochrome *c* reductase activity of homogenates and particulate fractions prepared from spleens of X-irradiated rats is consistent with numerous reports in the literature indicating that the activities of tissue respiratory enzymes (dehydro-

genases and cytochrome *c* oxidase) are little affected by *in vivo* irradiation at lethal levels.

Summary. Rats were subjected to 700 r of whole-body X-irradiation. At intervals of 13 to 85 hours after exposure, homogenates were prepared from spleens of irradiated and control animals and separated into 4 subcellular fractions by differential centrifugation. Per mg of nitrogen, DPNH cytochrome *c* reductase specific activity of the homogenates from X-rayed rats was increased significantly over the control levels at 13 and 37 hours after exposure. At 85 hours, the difference was much less owing to an increased specific activity of the enzyme in the control homogenates, as well. Expressed on the basis of nitrogen, cytochrome reductase specific activities of nuclear, microsomal, and supernatant fractions after irradiation showed no striking changes as compared to control values. Specific activities of the mitochondrial fractions from spleens of irradiated animals were decreased significantly as compared to control levels. The % of total DPNH cytochrome *c* reductase activity recovered in the 4 fractions prepared from the spleens of irradiated and control animals was essentially the same.

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Received February 7, 1957. P.S.E.B.M., 1957, v95.

Effects of Adrenergic and Cholinergic Drugs Injected by Intra-Carotid Route on Electrical Activity of Brain. (23112)

V. G. LONGO AND B. SILVESTRINI (Introduced by K. R. Unna)
Laboratory of Therapeutical Chemistry, Istituto Superiore di Sanitá, Roma

It has previously been reported(Longo, 10) that the EEG pattern provoked by intracarotid injection of acetylcholine (ACh) in rabbits is similar to the "activation" or "arousal" response produced by external stimuli. Similar observations have been made by Rinaldi and Himwich(15), who consider this effect as a specific action of cholinergic drugs on the reticular activating system. On the other hand, much experimental data suggest that cholinergic drugs are not the sole mediators of the activation response. In fact, it has been demonstrated that epinephrine and amphetamine are also able to elicit an activation picture in the EEG of experimental animals(Bonvallet *et al.*, 1; Elkes *et al.*, 6; Schallek and Walz, 17). This paper is concerned with the modifications of EEG after injection of acetylcholine, eserine, epinephrine, and amphetamine directly into the brain circulation.

Methods. Fourteen non-curarized unanesthetized rabbits and 12 "cerveau isolé" preparations (for details of "cerveau isolé" preparation in rabbit, *cf.* Longo and Silvestrini, 12) were used. Blood pressure was measured in majority of experiments, connecting femoral artery to mercury manometer. Some additional experiments were carried out on cats curarized with d-tubocurarine. The technic(Longo, 10) consists in cannulating the internal carotid in the rabbit so that the injected drug reaches the brain directly. Precautions were taken to avoid arousal reactions