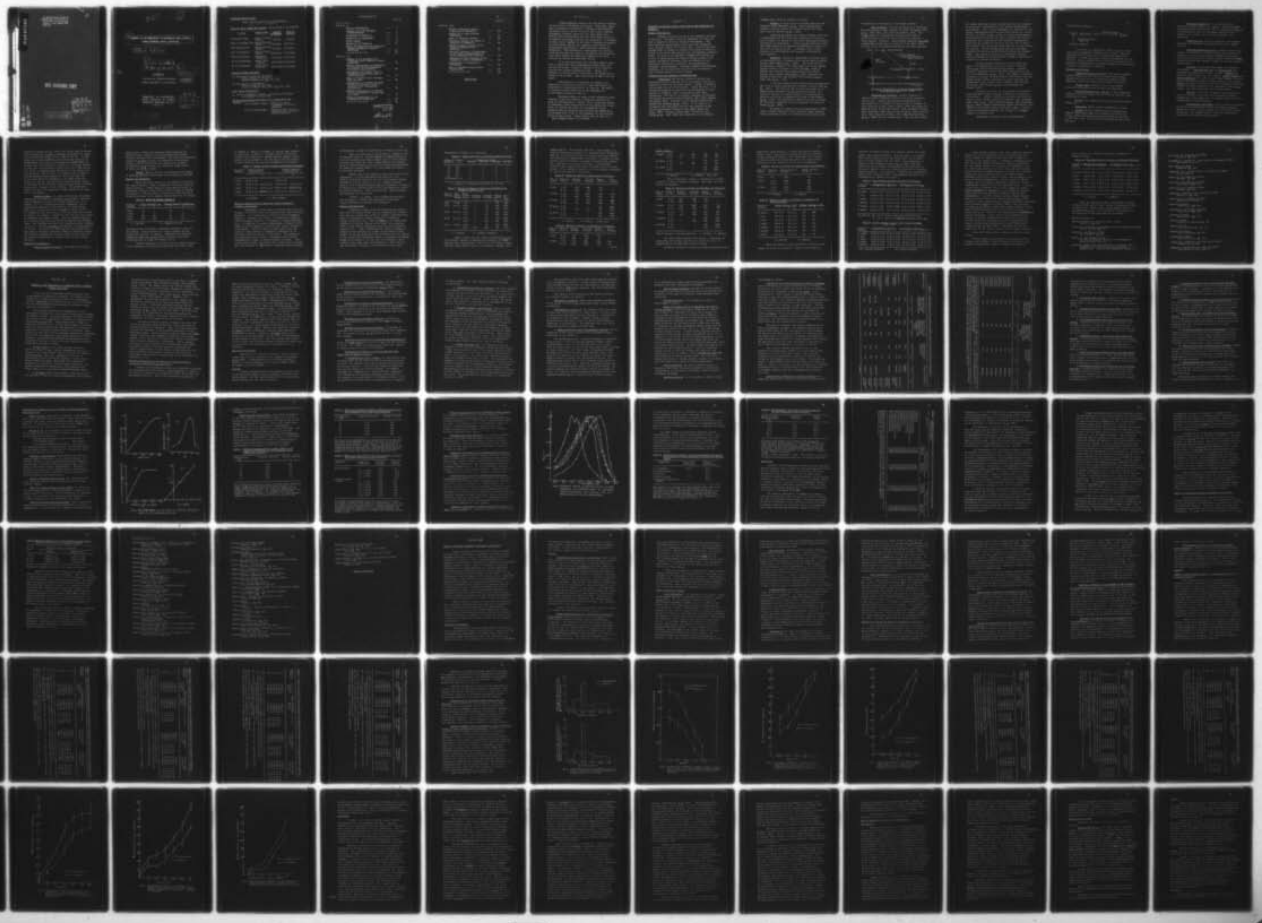
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(2) Metabolism of Carotenoids
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J.Sci.and Industr.Res.(India) 34, 219, 1975.

PAPER UNDER PREPARATION:

(1) Effect of hypobaric hypoxic conditions on lysosomes.
K.Thyagarajan and H.R.Cama

Two Research Fellows got their Ph.D. degree under the project:

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P r e f a c e

Living organisms interact not only with one another but also with a fluctuating environment. They must, therefore, continually accommodate to changes in environmental parameters such as, solar radiation, heat, cold, humidity, atmospheric pressure, pollution with toxic gases, etc. Besides these environmental factors, nutritional status of an organism profoundly influence its physiology. To preserve internal milieu constant in the face of changing environment and nutritional insults, the organism should respond by appropriated physiological, behavioural and biochemical adjustments. When alteration in environment or nutrition adversely perturbs the organism, biochemical mechanisms may ensue which restore the functional capacities to their previous or altered but stabilized levels.

It is very well known that vitamin A plays a major role in growth and fastly regenerating tissues apart from its role in vision. Vitamin A appears to be an essential nutrient for increased metabolic needs. Hence, the influence of various stress conditions on the metabolism of vitamin A was studied. This study forms the Part I of this report.

Carotenoids act as precursor of vitamin A and depending on provitamin A activity, these carotenoids are classified as biologically active or inactive. The effect of different stress conditions on the absorption of β-carotene/are reported in Part II.

Lysosomes contain an array of acid hydrolases which exhibits a voracious capacity to hydrolyze virtually all kinds of macromolecules. However, due to structural latency of lysosomes, normal constituents of cell escape the attack. Hence, we studied the effect of environmental stress condition such as simulated high altitude and regeneration on lysosomes with a view to understanding the repercursions caused by this stress. These studies form the Part III.

/and cleavage enzyme of β-carotene

P A R T - I

INFLUENCE OF VARIOUS STRESS CONDITIONS ON THE METABOLISM OF VITAMIN A.

General Introduction

Vitamin A constitutes one of the essential nutrients in the diet. Its absence leads to impairment of vision and lack of growth in the early stages of development. Only three essential physiological forms have been recognized, viz., retinol, retinal and retinoic acid. Liver is the chief storage organ in animals to preserve the excess of vitamin A formed in the system than required for the immediate metabolic needs. In spite of the extensive research work carried out so far, in establishing the relation between vitamin A and several biochemical processes (that are affected in its deficiency) like protein synthesis, mucopolysaccharide synthesis, steroidogenesis, cartilage integrity and rhodopsin synthesis, yet the molecular mode of action of vitamin A still remains obscure and quite elusive.

Vitamin A and its metabolic transformations

Absorption: The retinyl esters in the diet are hydrolyzed in the intestinal lumen by pancreatic lipase and hydrolase situated on the brush border. Retinol is absorbed into mucosal cell by active process. Inside the cell, it is re-esterified(mainly as palmitate) and transported to liver chiefly by lymphatic route in association with lymph chylomicron. The mode of absorption, mechanism of transport and storage have been reviewed [Ganguly, 1960, 1967; Ganguly and Murthy, 1970]. Retinoic acid is rapidly absorbed and metabolized [Deshmukh et al., 1964]. It is found to be conjugated with glucuronic acid and then excreted in the bile as glucuronide [Dunagin et al., 1965; Lippel and Olson, 1968]. Further, studies with ^{14}C-retinoic acid [Fidge et al., 1968, 1969] have shown that it is absorbed

through portal route in contrast to retinol.

Storage: In liver retinol is stored mainly as the palmitate ester [Mahadevan et al., 1961] although small amounts of retinyl oleate and retinyl stearate have also been detected [Goodman et al., 1965].

Linder et al.[1971] have prepared Kupffer cells free of hepatocytes and have shown only 4 percent of the total liver vitamin A is present in them while remaining 96 percent distributed in hepatocytes. Nyquist et al.[1971] have shown that Golgi bodies store highest concentration of retinol in the cells as retinyl esters. A possible role of Golgi bodies in the mobilization and transport of retinol has been suggested.

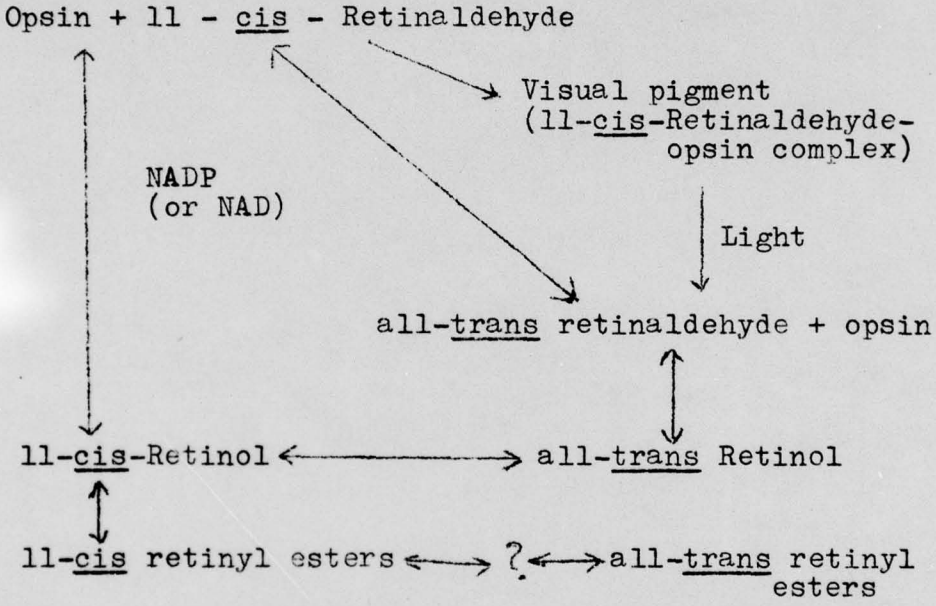
Transport: Retinol is transported to various tissue in association with a binding protein called Retinol-Binding Protein (RBP) [Kanai et al., 1968]. RBP interacts with pre-albumin of plasma in 1:1 molar ratio and in this PA-RBP complex retinol circulates in blood [Goodman, 1969a,b]. RBP has been isolated from animal species like rabbit, monkey, goat, chicken [Huang et al., 1972; Vahlquist and Peterson, 1972; Muto and Goodman, 1972; Peterson et al., 1973; Cama et al., 1976].

It has been observed that during vitamin A deficiency RBP level in serum decreases and increases in liver [Muto et al., 1972]. The levels of RBP in serum have shown to be significantly decreased during hypervitaminosis A. The vitamin A toxicity in hypervitaminosis A is attributed to delivery of retinol to membranes not bound to RBP [Mallia et al., 1975]. The secretion of RBP by the liver to plasma is efficiently regulated by the availability of vitamin A [Smith et al., 1973].

At present it appears that the significance of RBP seems to afford stability to the otherwise highly unstable retinol molecule as well as to facilitate the specific mode

of transfer to the receptor on the target tissues.

Role in Vision: The role of vitamin A in vision has been established by Wald, Hubbard and their colleagues [Wald et al., 1960; Wald, 1968; Wald and Hubbard, 1970]. The 11-cis isomer of retinaldehyde interacts with opsin, a protein present in the rod cells of retina to form the visual pigment, rhodopsin. Retinaldehyde undergoes cis-trans isomerization cycle with the proteins of the opsin family as shown by the following scheme during the visual process:



Cis-trans isomerization cycle of retinaldehyde in vertebrate eyes [Wald, 1968]

Metabolism of Vitamin A: Several congeners of vitamin A exert biological activities by their conversion into retinol. Alcohol dehydrogenase brings about the interconversion of retinal and retinol and the presence of this enzyme in various tissues [Bliss, 1949, 1951; Wald and Hubbard, 1949; Merritt and Tomkins, 1959; Zachman and Olson, 1961; Futterman, 1963; Dalziel and Dickinson, 1965; Koen and Shaw, 1966; Fidge and Goodman, 1967, 1968; Krishna Mallia et al., 1969a] has been demonstrated. Koen and Shaw [1966]

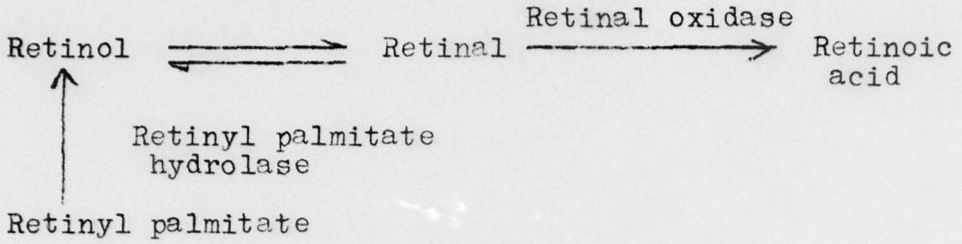
have shown that when soluble extracts of liver are analyzed by starch gel electrophoresis, five alcohol dehydrogenase bands appear. Four of these presumably are isozymes of liver alcohol dehydrogenase which oxidize both ethanol and retinol. However, the fifth enzyme, termed as retinol dehydrogenase 3, differs markedly from the rest and is specific for retinol. Similarly, they demonstrated three alcohol dehydrogenase bands from the extract of retina of the rat, two of which were specific for retinol and third could act on both alcohol and retinol. Retinol dehydrogenase from the intestinal mucosa of rat has been purified 13-fold [Fidge and Goodman, 1968]. This enzyme is relatively non-specific towards various aldehydes and requires NADH or NADPH as cofactor for reduction of retinal to retinol.

Retinyl esters, irrespective of the fatty acyl moiety, are hydrolyzed to retinol in the rat intestine [Gary et al.(1940); Mahadevan et al.(1963)]. Retinyl palmitate hydrolase activity has been demonstrated in the rat liver [Mahadevan et al., 1966]. The enzyme is primarily localized in nuclear and mitochondrial fractions of the cell. Though retinyl acetate hydrolase has been identified and characterized from various tissues [Olson, 1964; Bertram and Krisch, 1969; Mathur et al., 1970; Natanson and Eremina, 1972].

Retinoic acid which is even superior to retinol in promoting growth [Malathi et al., 1963], is not converted into retinol. However, retinal is converted into retinoic acid by liver aldehyde oxidase [Dmitrovsky, 1961; Futterman, 1962; Bamji et al., 1962; Mahadevan et al., 1962; Lakshmanan et al., 1964], alcohol dehydrogenase [Elder and Topper, 1961, 1962] and by xanthine oxidase [Futterman, 1962]. Aldehyde oxidase irriversibly oxidizes both 11-*cis* and all-*trans* retinal to retinoic acid.

Metabolism of vitamin A can be schematically

represented as follows:



It is very well known that vitamin A plays a major role in the maintenance of growth and in vision. When the metabolic needs are more as it happens when animals are subjected to stress conditions, vitamin A metabolism is also affected. Hence, studies were carried out to investigate the influence of hypothermia and ethanol feeding on the absorption, storage and metabolism of vitamin A.

Materials and Methods

Light petroleum (b.p.40-60°): The solvent supplied by ESSO Company was left over 10 percent aqueous $KMnO_4$ solution for three days with occasional shaking, washed, distilled, dried over $CaCl_2$ and redistilled before use.

Diethyl ether (Alembic and Co.) was freshly distilled over reduced iron to remove any peroxides.

Spectroscopically pure ethanol: It was prepared by refluxing rectified spirit with zinc dust and KOH for 6 hrs. distilling it twice.

Acetone: The commercial product was distilled twice before use.

Chloroform: Analar quality chloroform was used.

Alumina: Neutral alumina obtained from National Chemical Laboratory, Poona was refluxed with ethanol for 2 hrs., washed with light petroleum and dried in the oven at 120° for 6 hrs. It was partially deactivated with water and used.

Antimony trichloride: It was obtained from Albright and Wilson (Mfg.) Ltd.,London. Freshly prepared saturated solution (25 percent, w/v) of antimony trichloride in chloroform was used for Carr-Price reaction after the addition of 1 percent (v/v) of acetic anhydride just before use.

Vegetable oils: Refined, deodorized, decolourized groundnut oil obtained from Hindustan Lever Ltd., Bombay, was used.

Ethanolic hydrochloric acid: Hydrogen chloride gas, prepared by treatment of sodium chloride with concentrated sulphuric acid was passed through concentrated sulphuric acid and was bubbled into absolute alcohol (ethanol) to give suitable dilution with absolute ethanol to give the required concentration.

Thiourea was obtained from Hopkins and Williams Ltd.,London. Thiobarbutric acid and Triton X-100 were purchased from Sigma Co., St.Louis Mo.USA. Atebrine was purchased from George T.Gun Ltd.,London. Retinyl acetate and retinyl palmitate were generous gifts from Hoffman -La Roche, Basle, Switzerland through the kind courtesy of Dr.O.Isler. Retinal was prepared by the oxidation of synthetic crystalline retinol with MnO_2 according to the procedure of Ball et al. (1948). All other chemicals were of Analar grade.

Thiourea: Four grams of thiourea was dissolved in 100 ml of glacial acetic acid and the solution filtered through glass wool before use. The reagent could be stored indefinitely at room temperature.

Thiobarbutric acid (TBA): 600 mg of TBA was dissolved in 100 ml of spectroscopic ethanol and filtered through Whatman No.1 filter paper and stored in cold (0-5°C) before being used. The reagent could be kept for considerable period, but was always freshly prepared.

Vitamin A-deficient animals: Weanling male albino rats of the Institute strain weighing around 30-40 g were fed on a vitamin A-free diet, the percent composition of which is: casein (ethanol refluxed and ether extracted), 18; starch, 63; sucrose, 10; refined groundnut oil, 5; salt Mixture (Hawk and Oser, 1931), 4. The following vitamins were also added per kg of diet: calciferol, 100 µg; α-tocopheryl acetate, 100 mg; 2-methyl-1-4-naphthaquinone, 5 mg; thiamine hydrochloride, 5 mg; riboflavin, 5 mg; nicotinic acid, 50 mg; pyridoxin, 5 mg; pantothenic acid, 50 mg; biotin, 500 µg; folic acid, 500 µg; inositol, 100 mg; choline chloride, 1.0 g; ascorbic acid, 1.0 g. Aqueous solutions of water-soluble vitamins were prepared in suitable concentrations and requisite amount was mixed with diet every day. The basal ration was given to all the rats ad libitum with an adequate supply of fresh water. Generally, the rats maintained on this diet were depleted of vitamin A reserves within about 28 days. The rats were weighed at the same time at biweekly intervals for the first weeks and then every day until their weights reached a plateau stage. A rat was considered depleted of vitamin A when its net gain in weight on four successive days did not exceed 1 g, provided that on two of the days the animal did not gain in weight [Bliss and György, 1951]. At the depletion period the rats invariably reached a constant weight of 80-90 g on an average. Control rats received 2 mg vitamin A in orally weekly apart from the above specified vitamin A-free diet.

Simulation of hypothermia: Vitamin A-deficient rats were kept in cooler (0-5°C) for different interval of time while control animals are vitamin A-deficient but kept at room temperature about 28°C.

Homogenization and preparation of tissue fractions: Vitamin A-deficient rats were given high oral dose of vitamin A and exposed to simulated high altitude for a period of 4 hrs. Animals were killed under light ether aneasthesia.

Liver, intestine, kidney and stomach were dissected out, freed from adhering tissues and washed in cold physiological saline (0.9 percent). The tissues were dried between the folds of filter paper, weighed and immediately kept in a beaker in an ice bucket. In the case of livers, a part weighing about 1 g was cut and kept separately for the determination of stored vitamin A. The rest of the liver was used for homogenization.

Homogenization of livers: The livers were minced into small pieces and homogenized in a Potter-Elvehjem Homogenizer in 0.25M sucrose (1:5 w/v). The homogenate was first centrifuged at 1000xg for 10 min to separate the nuclear fraction. The supernatant was centrifuged at 6500xg for 10 min to remove the mitochondrial fraction and the post mitochondrial supernatant was centrifuged at 12000xg for 30 min in a Sorvall RC 2B Centrifuge. The supernatant thus obtained was spun at 100000xg for 60 min in Spinco L Model Beckman Ultracentrifuge. The supernatant was used to assay retinal oxidase and reductase.

Enzyme Assays

Retinal reductase: The assay system consisted of 0.5 µmoles of retinal in 0.1 ml alcohol, 0.1 ml of $NADH_2$ (1µ mole), 0.1 ml reduced glutathione (2µmoles), 0.3 ml atebrine 3mmoles and 0.5 ml enzyme. The whole volume was made upto 3 ml with 0.1 M sodium phosphate buffer, pH 7.2. Atebrine was employed as inhibitor of retinal oxidase activity [Lakshmanan et al., 1964]. The incubation was carried out at 37°C for 60min and the reaction was stopped by the addition of 5 ml ethanol. The reaction mixture was then extracted three times with 25 ml of distilled peroxide-free diethylether. The extracts were combined, evaporated to dryness in vacuo and dissolved in a few ml of light petroleum. The reaction products were separated by adsorption on 5 percent (v/w)-water-deactivated alumina column. The column was developed with 2 percent (v/v) diethylether in light petroleum to remove

uncoverted retinal and the retinol was eluted with 25 percent (v/v) diethylether in light petroleum and estimated in light petroleum from its $E^{1\%}_{1\ cm}$ value of 1650 at 325 nm. In some assay, the reaction substrate left behind was estimated by thiobarbutric acid assay of Futterman and Saslaw [1961] as follows: The test solution was diluted to 3 ml water ethanol. To each tube 1 ml of thourea reagent and 1 ml of TBA reagent were added. A reagent blank containing 3 ml of ethanol was also prepared. All the tubes were shaken so as to achieve uniform mixing of the reagents and left in the dark for 30 min after which the absorbance of the pink colour was measured at 530 nm. The difference between retinal content of the blank of the reductase enzyme and that of the reaction mixture gave a measure of the activity. The calibration curve for retinal was constructed using retinal in the range of 10 to 60 nmoles.

Retinal oxidase: The enzyme activity was determined essentially according to Lakshmanan et al.[1964]. The assay system consisted of 1µmole of retinal suspended in 0.1 ml ethanol, 0.1 ml of reduced glutathione (2µmoles), 0.5 ml enzyme(containing above 5 mg protein), the volume being made upto 3 ml with 0.1M sodium phosphate buffer, pH 7.8. The incubation was carried out at 37°C for 30 min and stopped by adding 5 ml ethanol. A blank was done with the same amount of substrate but without enzyme. Both the reaction mixtures were extracted with 25 ml of distilled peroxide-free diethyl-ether. The extracts were dried over anhydrous sodium sulphate, evaporated to dryness in vacuo and dissolved in known quantity of ethanol. The retinal in blank and test was estimated by TBA assay of Futterman and Saslaw [1961] as described earlier. The difference between the retinal amounts of the blank and the reaction mixture gave a measure of the retinal oxidase activity.

Estimation of Vitamin A

Liver, stomach and kidney: The tissue was cut into

small pieces, ground with anhydrous sodium sulphate and extracted with a mixture of light petroleum and freshly distilled peroxide-free diethylether. The ether was completely evaporated *in vacuo* and was then suspended in chloroform. Vitamin A content of solution was estimated by antimony trichloride colour reaction, the absorbance being measure at 620 nm and $E^{1\%}_{1\ cm}$ of 4800.

Protein: Protein content was measured by the method of Lowry *et al.*[1951] using bovine serum albumin as standard.

Results and Discussion

Liver and plasma vitamin A were estimated in control and exposed animals. Table 1 depicts the levels of liver and plasma vitamin A. Plasma vitamin A level gives an index of mobilizable vitamin A while that of liver an index of storage of vitamin A. From the Table, it can be seen that the liver vitamin A is lower in the exposed animals than that of control.

Table-1: **Liver and Plasma Vitamin A**

Period of exposure	Liver vitamin A µg		Plasma vitamin A µg/100 ml	
	C	E	C	E
0 Week	34	-	38	-
1 Week	x	x	x	x
2 Week	286	215	48	44
3 Week	454	345	43	41

C = Control E = Exposed to cold

This may be due to decreased absorption and/on the increased utilization. Plasma vitamin A of exposed animals did not differ much from that of control. Sundaresan and Theriault [1968] and Sundaresan *et al.*[1965] reported an increased utilization and no change in vitamin A level.

Retinal reductase and oxidase are the enzymes involved in the conversion of vitamin A alcohol form into active form

of vitamin A. Effect of exposure to cold on these enzymes is represented in Table 2. Specific activity of retinal reductase was unaltered in exposed animals, whereas retinal oxidase levels increased. The increase in retinal oxidase activity indicates the increased utilization of vitamin A.

Table-2: Hepatic Retinal Oxidase and Retinal Reductase

Period of exposure	Retinal Oxidase (Sp.activity)		Retinal Reductase (Sp.activity)	
	C	E	C	E
0 Week	12.34 ± 0.2	-	9.46 ± 0.8	-
1 Week	13.97 ± 0.8	15.20 ± 0.4	9.82 ± 1.5	9.72 ± 2.0
2 Week	14.37 ± 1.1	17.37 ± 1.6	10.34 ± 1.3	10.30 ± 0.4
3 Week	13.85 ± 0.8	16.20 ± 1.3	10.12 ± 0.8	10.32 ± 0.7

C = Control E = Cold exposed

Effect of short-term and long-term ethanol feeding on vitamin A metabolism.

Irrespective of the forms of retinyl esters fed, the vitamin A is stored in the liver mainly in the form of retinyl palmitate. It is converted to retinol by retinyl palmitate hydrolase. Retinol, thus formed, is oxidized to retinal by retinal reductase which is an isoenzyme of alcohol dehydrogenase. In addition to alcohol dehydrogenase, microsomal mixed function oxidase system of liver can also oxidise ethanol to acetaldehyde. Lieber and Decarli [1970] showed that the levels of these enzymes are changed in adaptation to the increased influx of ethanol in the system. This adaptation may indirectly affect vitamin A metabolism since alcohol dehydrogenase is known to oxidize retinol in liver. Hence, in the present study, experiments were carried out to investigate the influence of short-term and long-term ethanol feeding

on absorption, storage and metabolism of vitamin A in the rat.

Rats were made vitamin A-deficient by feeding a diet as mentioned earlier. Various methods of ethanol-feeding to rats were examined like mixing ethanol with the diet, making a slurry and feeding the rats, mixing ethanol with drinking water or forece-feeding the rats with different concentrations of ethanol by stomach tubing. The last method was found to be the most convenient.

Female rats of the Institute strain were used for the experiments. In some experiments male rats were used. Vitamin A-deficient rats were fed 2 ml of 25 percent ethanol followed by 2 mg of vitamin A in oil. Control rats were given 2 ml of isocaloric sucrose solution followed by vitamin A. At various time intervals four rats of each group were killed. by ether anaesthesia. Blood was collected. Liver, stomach and intestine were dissected.

Vitamin A in plasma, liver, stomach and intestine were estimated. Retinyl ester hydrolase of intestine, retinal reductase and oxidase were estimated.

Results and Discussion

During the course of the experiment it was noticed that rats fed ethanol became vitamin A-deficient at a faster rate than the control rats reared on vitamin A-deficient diet. Table 1 represents the dosage tolerance of ethanol by vitamin A-deficient rats. From the table, it is evident that feeding of 50 percent ethanol is too toxic to be ingested. Hence, in the present experiments rats were fed 25 percent ethanol (w/v). Table 2 represents the effect of dosage of ethanol on the absorption of vitamin A when 5000 I.U./rat vitamin in oil was given along with different doses of ethanol, it was evident that intestinal vitamin A increases with dosage though at 50 percent it is less than at 35 percent. Liver storage on the other hand, shows depletion. This indicates that absorption of vitamin A is somewhat affected, while

utilization of vitamin A is increased.

Table-1: Dosage tolerance by vitamin A-deficient rats

Dosage of ethanol % wt/vol	1 ml	Number of deaths 1st day	2nd day	3rd day	4th day
10	(5)δ	x	x	x	x
25	(5)	1	x	x	x
35	(5)	1	2	x	x
50	(8)	3	2	x	x
30 % sucrose	(4)	x	x	x	x

δ - Figures in parenthesis indicate no. of rats.

Table-2: Effect of dosage of ethanol on absorption of vitamin A (female rats).

Dose of ethanol	Time after dosage	Group of rats number	Stomach IU/organ	Intestine IU/organ	Plasma IU/100 ml	Liver IU/ organ
0 %	0 hrs.	(3)	-	-	130	312
10 %	24 hrs.	C(4)	-	60	215	2400
		A(5)	-	84	220	2000
25 %	24 hrs.	C(5)	-	73	195	2370
		A(5)	-	94	228	1820
35 %	24 hrs.	C(4)	-	75	207	2500
		A(4)	-	160	192	1481
50 %	24 hrs.	C(5)	-	48	210	2435
		A(4)	125	110	161	460

C = Control A = Alcohol (ethanol)

Tables 3 and 4 show the effect of short-term feeding of 25 percent ethanol on absorption of vitamin A in female and male rats, respectively. After 1 hr. of feeding, stomach contains around 4000 IU whereas in stomach of control, only

around 1000 IU. It decreases with time. More vitamin A is found in intestine of control at 1 hr.period than ethanol-fed. With time it is depleted faster in control rats than ethanol-fed indicating the absorption is affected in ethanol-fed rats. Plasma levels of vitamin A is higher in control than ethanol-fed. Moreover, as a result of vitamin A feeding plasma levels go up in control and return to steady level at 24 hrs. period. Liver storage of vitamin A is very markedly influenced in control rats than the ethanol-fed.

Table-3: Effect of ethanol on absorption of vitamin A

Time of dosage	Group of rats No.	Stomach IU/organ	Intestine IU/organ	Plasma IU/100 ml	Liver IU/organ
1 hour	C (4)	911	3296	200	-
	E (4)	4120	85	73	-
3 hours	C (4)	113	1985	520	250
	E (3)	1220	1100	284	120
6 hours	C (4)	65	342	560	825
	E (4)	420	830	480	532
12 hours	C (3)	-	-	410	1485
	E (3)	120	400	435	1026
24 hours	C (4)	-	-	186	1852
	E (4)	-	-	192	1437

C = Control E = Ethanol Female Rats

Table-4: Effect of ethanol on absorption of vitamin A

Time of dosage	Group of rats No.	Stomach IU/organ	Intestine IU/organ	Plasma IU/100 ml	Liver IU/organ
1 hour	C (4)	856	2964	282	-
	E (4)	4023	120	64	-
3 hours	C (4)	113	1692	593	264
	E (3)	1344	1023	290	115

contd...

contd...Table-4

6 hours	C (4)	51	328	608	740
	E (3)	414	941	515	643
12 hours	C (4)	-	-	430	1337
	E (4)	85	382	441	953
24 hours	C (4)	-	-	195	1690
	E (4)	-	-	190	1365

C = Control E = Ethanol Male rats

Table 5 represents ethanol on absorption of vitamin A when it is fed in aqueous dispersion. Here also, the same pattern is observed.

Table-5: Effect of ethanol on absorption of vitamin A

Time of dosage	Group of rat No.	Stomach IU/organ	Intestine IU/organ	Plasma IU/100 ml	Liver IU/organ
1 hour	C (4)	870	3348	220	-
	E (4)	4235	98	78	-
3 hours	C (4)	150	1865	540	300
	E (4)	1320	1050	260	95
6 hours	C (4)	72	285	545	925
	E (4)	405	905	468	510
12 hours	C (4)	-	-	385	1562
	E (4)	-	360	400	1100
24 hours	C (4)	-	-	195	1930
	E (4)	-	-	190	1480

5000 IU vitamin A aqueous dispersion C = Control E = Ethanol

The above tables indicate that vitamin A absorption is lowered but at the same time vitamin A levels in liver are depleted faster on short-term ethanol feeding.

On chronic feeding of ethanol to vitamin A-deficient rats

showed that liver vitamin A is lower than that of control. Tables 6 and 7 represent the content of liver and plasma on chronic feeding of ethanol. This study shows that feeding of ethanol increases utilization only in the intial stages whereas after one month this effect is not pronounced.

Table-6: Effect of ethanol on hepatic stores of vitamin A

Days on ethanol	Group of rats No.	Liver vitamin A IU/organ	Plasma vitamin A IU/100 ml
0 Days	(3)	1680	164
7 Days	C (4)	1596	168
	E (4)	1348	200
15 Days	C (4)	1548	156
	E (4)	1300	192

C = Control E = Ethanol

Table-7: Effect of ethanol on storage of vitamin A on chronic feeding.

Period of feeding	Liver vitamin A(IU)		Plasma vitamin A (IU)	
	C	E	C	E
24 hours	291 ± 19	262 ± 18	128	169
1 week	328 ± 15	217 ± 13	134	174
2 weeks	342 ± 37	262 ± 24	140	151
1 month	368 ± 29	281 ± 14	128	126
3 months	342 ± 17	312 ± 26	137	138
5 months	408 ± 22	388 ± 26	134	118
6 months	474 ± 19	413 ± 10	141	105

C = Control E = Ethanol

Effect of ethanol feeding on vitamin A metabolism was studied in liver and intestine. Tables 8 and 9 show the

influence of chronic feeding of 25 percent ethanol on retinal oxidase and retinal reductase. From the table it is evident that retinal reductase level in ethanol-fed rats is higher only in initial stages till one month. This observation is similar to the findings of Dajani et al.[1963, 1965] who showed that liver alcohol dehydrogenase levels increased in rats given ethanol as the sole drinking fluid for 24 weeks(25 percent solution). This increase in ADH(Alcohol dehydrogenase) activity was consistent with a corresponding increase in the accumulation of lipids in the liver.

Table-8: Liver Retinal Oxidase and Retinal Reductase

Period of feeding	RR Specific Activity		RO Specific Activity	
	C	E	C	E
24 hours	10.30 ± 0.30	10.42 ± 0.24	13.66 ± 0.32	13.66 ± 0.30
1 week	10.92 ± 0.31	13.46 ± 0.19	13.40 ± 0.72	14.36 ± 0.33
1 month	11.33 ± 0.46	13.02 ± 0.24	12.30 ± 0.65	13.68 ± 0.45
3 months	11.80 ± 1.03	11.26 ± 0.50	12.40 ± 0.36	13.98 ± 0.26
5 months	11.41 ± 0.23	9.76 ± 0.18	13.12 ± 0.35	11.25 ± 0.34
6 months	10.89 ± 0.33	8.07 ± 0.25	13.36 ± 0.31	10.53 ± 0.94

Sp.Activity. RR = µg of retinol formed/mg protein/60 min.
RO = µg of retinal disappearing/mg protein/30 min.

Table-9: Hepatic Retinal Oxidase and Retinal Reductase
(Male rats)

Period of feeding	RR Specific Activity		RO Specific Activity	
	C	E	C	E
24 hours	10.85 ± 1.30	10.35 ± 1.57	12.45 ± 1.30	13.70 ± 1.33
1 week	11.21 ± 1.40	14.33 ± 0.82	13.35 ± 0.70	14.80 ± 1.20
1 month	11.84 ± 0.31	11.97 ± 1.30	12.44 ± 0.20	14.53 ± o.27
3 months	12.33 ± 0.85	11.66 ± 1.10	12.51 ± 0.54	14.11 ± 0.17
6 months	11.48 ± 0.90	7.83 ± 0.90	12.81 ± 0.82	10.21 ± 0.30

RR = Retinal Reductase RO = Retinal Oxidase

After feeding ethanol to rats for 1 month the observed increase in retinal reductase became apparently less and after 6 months of ethanol feeding there was a significant decrease in the enzyme levels. A possible explanation may be that apart from alcohol dehydrogenase which is responsible for the hepatic oxidation of ethanol to acetaldehyde, there are other enzyme systems which also carry out this function. Lieber and Decarli [1971] observed that there is an adaptive increase in the activity of hepatic microsomal ethanol oxidizing system after ethanol feeding, with no change in the level of alcohol dehydrogenase. After 24 days of ethanol feeding, they even found a significant decrease in alcohol dehydrogenase levels in liver of ethanol-fed rats. A decrease in the retinal reductase levels after 6 months of chronic ethanol ingestion is possibly due to the fact that damaged liver cells may have decreased ability to form alcohol dehydrogenase. A similar decrease in the alcohol dehydrogenase level was found in rat liver [Dajani et al., 1965] and also in liver samples taken by biopsy from human alcoholics with cirrhosis [Figueroa and Klotz, 1962]. More recently, van Thiel et al.[1974] reported that ethanol inhibited testicular retinal production and that the relative vitamin A deficiency may be a factor in the pathogenesis of sterility in chronic alcoholics. Recent studies have demonstrated the presence of alcohol dehydrogenase activity in the retina, and have shown that alcoholics experience night-blindness because of competitive inhibition of retinol formation by ethanol [Mezey and Hold, 1971]. Rats given one dose of ethanol for 24 hrs. had no changes in their enzyme activity.

These above tables show no change in retinal oxidase levels.

Table 10 gives the activities of intestinal retinal reductase and oxidase. From the table, it is evident that alcohol feeding leads to reduced specific activities of

these enzymes, the reduction being more pronounced on chronic feeding.

Table-10: Intestinal Retinal Oxidase and Retinal Reductase

Period of feeding	RR Specific Activity		RO Specific Activity	
	C	E	C	E
24 hours	5.16 ± 0.12	5.49 ± 0.22	5.10 ± 0.14	5.12 ± 0.74
1 week	5.01 ± 0.38	4.72 ± 0.32	5.16 ± 0.32	4.55 ± 0.16
2 weeks	5.30 ± 0.17	4.57 ± 0.17	5.33 ± 0.11	4.30 ± 0.10
1 month	5.37 ± 0.10	5.08 ± 0.21	5.41 ± 0.18	5.04 ± 0.10
3 months	5.52 ± 0.12	4.50 ± 0.17	5.37 ± 0.16	4.38 ± 0.17
6 months	5.49 ± 0.17	4.67 ± 0.12	5.50 ± 0.21	4.88 ± 0.17

C = Control E = Ethanol

Thus, the findings of these experiments on ethanol feeding to rats indicate that vitamin A metabolism in liver is affected by ethanol intake, though perhaps only as a secondary consequence of the adaptation of the animal to the ingestion of high concentrations of ethanol.

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P A R T - II

CHANGES IN THE METABOLISM OF β-CAROTENE UNDER DIFFERENT STRESS CONDITIONS

It is very well established that carotenoids serve as precursors of vitamin A in animals. The carotenoids are classified as either biologically active or biologically inactive depending on the ability to give rise to vitamin A. The most biologically active precursor of vitamin A known uptil now is β-carotene.

Moore [1930] reported the accumulation of large quantities of vitamin A in liver upon feeding massive amounts of carotene to rats. The first suggestion that the intestine was involved in the conversion of carotene to vitamin A came from Sexton *et al.*[1946]. They observed that vitamin A-deficient rats survived on feeding small amounts of carotene while the vitamin A-deficient rats maintained on parenteral injections of colloidal carotene died of deficiency. They pointed out that their results strongly suggested the intestine as the site of conversion. Using uniformly labelled ^{14}C-β-carotene, Olson [1961] demonstrated its conversion in rat intestine to retinol, retinyl ester and a fraction chromatographically similar to retinal.

In vivo studies with doubly labelled β-carotene [U-^{14}C and 15,15'^{3}H] have clearly demonstrated that it is cleaved at the 15,15'-double bond to two moles of vitamin A [Goodman *et al.*, 1966 a,b]. Analogous to the use of peroxidative agents in chemical cleavage of carotenoids to retinal [Barua and Barua, 1966,1969], the cleavage of β-carotene by intestinal sections occurs aerobically. The cleavage could not be demonstrated either anaerobically [Olson, 1961] or at low oxygen tension [Harashima, 1964].

In *in vitro* studies with isolated enzyme system from rat intestine it has been clearly demonstrated that β-carotene

is cleaved at the 15,15'-double bond to two moles of retinal [Olson and Hayashi, 1965; Goodman and Huang, 1965; Goodman et al., 1967]. The enzyme systems like retinal oxidase and retinal reductase which can further utilise the retinal formed, have also been found to be present in the rat intestinal mucosa [Mahadevan et al., 1962; Crain et al., 1967; Fidge and Goodman, 1968; Moffar et al., 1970]. Carotene 15,15'-dioxygenase has also been identified in the soluble non-particulate fractions of rat liver and kidney [Olson and Hayashi, 1965]. It has been partially purified from the intestinal mucosa of a few animal species [Fidge et al., 1969; Lakshmanan et al., 1968, 1972; Olson, 1969]. The enzyme has been shown to be inhibited by iron chelating agents and sulfhydryl inhibitors. The proper dispersion of the substrate (β-carotene) is very essential for its activity.

The above literature survey envisages a key role for intestinal carotene 15,15'-dioxygenase in the conversion of dietary carotene into vitamin A. Hence, it became imperative to study the influence of environmental parameters on this particular enzyme. As this enzyme is present along with other enzymes involved in the vitamin A metabolism, like retinal oxidase, it is impossible to discern the extent of conversion using unlabelled β-carotene as substrate. Hence, attempts were made to purify carotene 15,15'-dioxygenase from rabbit and guinea pig and to test the extent of conversion and relative specificities of this enzyme towards β-carotene and other substituted carotenoids. Attention was devoted to investigate the influence of hypothermia on the absorption of β-carotene in rat.

Partial purification and properties of carotene 15,15'-dioxygenase from guinea pig intestines.

Vitamin A is biosynthesized from its carotenoid precursors and depending upon their ability of transformation to vitamin A they have been divided into biologically active carotenoids and biologically inactive. With a few exceptions, biologically

active carotenoids possess atleast one intact β-ionone ring and fully conjugated isoprene side chain. β-Carotene and its various isomers like α-carotene and γ-carotene, apo-β-carotenals, 5,6- and 5,8-epoxides of β-carotene and cryptoxanthin are transformed to vitamin A in the rat [Moore, 1957; Glover, 1960; Subbarayan et al., 1966; Jacob John et al., 1970; Jacob John and Cama, 1972]. Anhydrolutein is transformed to 3-dehydrovitamin A in chick [Budowski et al.,1963], mouse [Budowski and Gross, 1965], rat [Savithry et al., 1972] and in fish [Gross and Budowski, 1966]. In fish astaxanthin (3,3'-dihydroxy β,β-carotene, 4,4'-dione) is converted to retinol [Grangaud et al., 1962, 1964]. These studies indicate the involvement of dioxygenase in cleaving these compounds to vitamin A. The isolated carotene 15,15'-dioxygenase from the rabbit intestinal mucosa has been shown to cleave α-, and β-carotene, 3,4, 3',4'-tetra-dehydro-β,β-carotene and few other carotenoids [Olson, 1969]. However, our knowledge regarding in vitro cleavage of naturally occurring ring substituted carotenoids is scanty. Therefore, in vitro studies were carried out to get an insight into the mode of transformation of carotenoids to vitamin A. Here, the partial purification of this enzyme from intestinal mucosa of rabbit and guinea pig, its properties and identification of products have been described.

Materials and Methods

Materials are as described earlier. Active manganese dioxide was prepared according to the method of Attenburrow et al.(1952). Monoperphthalic acid was freshly prepared whenever required according to the method of Böhme [1955] and Royals and Harrel [1955].

Methods

Retinal, 3-dehydroretinal and epoxides of retinal were estimated by TBA colour reaction according to Futterman and Saslaw [1961]. The TBA colour products of these respectively absorb maximally at 530, 550 and 470 nm.

Preparation of 5,6-epoxy-β-carotene: The method used for the preparation and purification of 5,6-epoxy-β-carotene was the same as reported by Subbarayan et al.[1966].

Preparation of 5,8-epoxy-β-carotene: It was synthesized from 5,6-epoxy-β-carotene by treatment with ethanolic-HCl. It was purified and crystallized as described by Jacob John and Cama [1972].

Preparation of 5,8,5',8'-diepoxy-β-carotene: 5,6,5',6'-Diepoxy-β-carotene was prepared by the action of monoperphthalic acid on β-carotene according to the procedure of Subbarayan [1966]. This compound on treatment with ethanolic-HCl resulted in 5,8,5',8'-diepoxy-β-carotene.

Preparation of 5,6-epoxy-α-carotene: 5,6-Epoxy-α-carotene was synthesized according to the method of Kishore et al.[1970].

Preparation of 5,8-epoxy-α-carotene: 5,8-Epoxide of α-carotene was prepared by treating 5,6-epoxide with ethanolic-HCl. The method of Kisore et al.[1970] was used to purify this compound.

Isolation and purification of lutein and anhydrolutein: Leaves of Tecoma stans were used as a source for the isolation of lutein. The method of Savithry et al.[1972] was employed to purify lutein and prepare anhydrolutein.

Purification of carotene 15,15'-dioxygenase from guinea pig and rabbit intestine.

1. Preparation of crude extract: Two adult guinea pigs (weighing about one kg each) were killed by giving a blow on the head. The intestine was removed quickly and washed with cold saline solution. It was cut open along the mesenteric line and the mucosa was carefully removed. The mucosal scrapings were homogenized in a Potter-Elvehjem homogenizer in 8 volumes of 0.1M potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 20,000xg for 15 min. The precipitate was discarded and the supernatant was designated

as crude extract. The crude extract contained 10-12 mg of protein/ml.

2. Treatment with protamine sulphate: The crude extract was subjected to protamine sulphate treatment to remove the nucleic acid material from the preparation. For every 8.0 ml of crude extract 0.1 ml of 2 percent protamine sulphate was added slowly by gentle stirring. After 10 min., the precipitate was removed by centrifugation at 20,000xg for 10 min and the supernatant was processed further.

3. Ammonium sulphate fractionation: Carotene 15,15'-dioxygenase from protamine sulphate supernatant was purified by ammonium sulphate fractionation. The supernatant was saturated to 25 percent with $(NH_4)_2SO_4$ and the precipitate formed within 15 min was centrifuged at 20,000xg for 10 min. The saturation of the supernatant was raised to 55 percent by the addition of solid $(NH_4)_2SO_4$ with gentle stirring. After 30 min., the precipitate was centrifuged at 20,000xg for 10 min. Carotene 15,15'-dioxygenase activity was found in the $(NH_4)_2SO_4$ precipitate (25-55 percent). This precipitate was designated as $(NH_4)_2SO_4$ pellet. The enzyme could be stored as $(NH_4)_2SO_4$ pellet at -20°C for a considerable period of time without appreciable loss of activity.

4. Acetone fractionation: The $(NH_4)_2SO_4$ pellet was dissolved in 0.1M potassium phosphate buffer (pH 7.8) to give a final protein concentration of 13-14 mg/ml chilled acetone (-20°C) was added to it to obtain the final concentration of acetone to 45 percent. The mixture was immediately centrifuged at 10,000xg for one min. at -10°C. The precipitate was designated as 0-45 percent acetone precipitate and found to be devoid of carotene 15,15'-dioxygenase activity. The precipitate was discarded and the protein from 45 percent acetone supernatant was precipitated further by the addition of chilled acetone (-20°C) to increase the final concentration of acetone 65 percent. The precipitate was centrifuged at 10,000xg for one min. at -10°C.

The precipitate obtained at this step contained carotene 15,15'-dioxygenase activity. It was immediately dissolved in 0.1M tris-HCl buffer (pH 8.5) and centrifuged at 20,000xg for 10 min to remove the insoluble proteins. The clear supernatant was used for all studies.

The carotene 15,15'-dioxygenase from rabbit intestine in a similar fashion, was studied.

Estimation of protein: Protein was estimated according to the method of Lowry et al.[1951] using bovine serum albumin as standard.

Dispersion of substrates: The substrates used for enzyme reaction were first dissolved in light petrol. To this 0.1 ml of Tween-20 was added/200 nmoles of substrates. The solution was evaporated under nitrogen. Adequate volume of glass distilled water was added to make the final concentration of substrates to 200 nmoles/ml. The substrates were then finely dispersed in water by sonication for 2 min.

5. Assay of carotene 15,15'-dioxygenase activity: All the conditions of this enzyme activity were standardized using β-carotene as the substrate.

The incubation mixture consisted 200 nmoles of substrate, 10 mg of GSH, 1 x 10^{-3}M of ferrous ammonium sulphate (final concentration), 10 ml of 0.1M tris-HCl buffer, pH 7.8 for assay of rabbit enzyme and pH 8.5 for guinea pig enzyme, 5-15 mg of enzyme protein and the total volume of incubation mixture was always made upto 25 ml with glass distilled water. The reaction was initiated by the addition of enzyme. An enzyme blank was taken as control for each substrate tested. The incubation was carried out in amber coloured flasks 100 ml at 37°C for 3 hrs. in the dark. After the incubation period, the reaction was stopped by the addition of 25 ml of acetone. The reaction mixture was extracted thrice with 50 ml aliquots of ether. The ethereal extract was washed with water, dried over anhydrous sodium suphate, evaporated under reduced pressure.

It is redissolved in light petrol and the product(s) was separated from their corresponding substrate.

Unit of enzyme activity: One unit of enzyme activity is defined as the amount of protein required to catalyze the formation of one nmole of retinal/hr. under standard assay conditions.

Specific activity: It is expressed as units of activity/mg of protein.

Assay of enzymes capable of degrading the product formed (retinal): Since the product formation was taken as the parameter of dioxygenase activity, other enzymes for which retinal can serve as the substrate were checked by assaying retinal disappearance by the different purified preparations of dioxygenase from guinea pig. The standard assay conditions for disappearance of retinal were the same as described for carotene dioxygenase enzyme except that the reaction was carried out for 1 hr., i.e., the linear range of this enzyme activity. A total reaction mixture of 2.5 ml consisted of 100 nmoles of retinal (dissolved in 0.1 ml of ethanol), 1.0 mg of GSH, 0.5 ml of enzyme preparation, 1.0 ml of 0.1M tris-HCl buffer (pH 8.5 for guinea pig enzyme and pH 7.8 for rabbit enzyme) and total volume was made upto 2.5 ml with glass distilled water. An enzyme blank was taken as the control for every reaction mixture. After an incubation period of 1 hr. at 37°C the reaction was stopped by the addition of 2.5 ml of ethanol. One ml aliquot was taken for the estimation of retinal by TBA method. The difference between the amount of retinal present in blank and in experiment was taken as the amount of retinal disappeared.

Unit of activity: One unit of enzyme activity is defined as the amount of protein required to catalyze the disappearance of one nmole of retinal/hr. under the assay conditions described.

Specific activity: It is expressed as units of enzyme

activity/mg of protein.

Separation and characterization of reaction products: After the reaction was stopped with acetone, the reaction mixture was extracted thrice with ether, dried over anhydrous sodium sulfate, evaporated to dryness in vacuo. It was redissolved in a small volume of petrol and applied quantitatively on thin layer chromatography (t.l.c.) plates coated with basic alumina. The authentic samples of expected products were also spotted side by side and the plate was developed using 3 percent (v/v) acetone in light petroleum. The area corresponding to the expected products was then scraped from t.l.c. plates, extracted with acetone and evaporated to dryness in vacuo. It was redissolved in light petroleum and their spectra were recorded on Cary-14 Recording Spectrophotometer. The absorption maxima of reaction products in light petroleum were represented in Table-1.

The reaction products were further characterized as their corresponding alcohols. The isolated products were treated with $LiAlH_4$ at -10° for five min in ether solution. The reaction was stopped by the addition of ice-cold water. The ether extract was evaporated to dryness, redissolved in light petroleum and spectra of reaction products were recorded. The absorption maxima (in light petroleum) of the products after reduction with $LiAlH_4$ are also given in Table-1.

The reaction products separated on t.l.c. were dissolved in spectroscopic ethanol (1 ml) and reacted with thiourea (1 ml) and TBA reagent (1 ml). The color was developed at room temperature in dark for 30 min and the spectra of TBA complexes were also recorded. The absorption maxima of TBA-complexes with different products are given in Table-1. Retinal, α-retinal, 5,6- and 5,8-epoxides of retinal, 3-hydroxyretinal and 3-dehydroretinal were thus identified as enzymatic products when their corresponding substrates were used.

Quantitative estimation of reaction products: The product(s) formed from various carotenoids was quantitatively

Table-1: Identification of reaction products from different carotenoid substrates

Substrate used	Absorption max. (nm) in light petroleum of products formed		Absorption max. (nm) in light petroleum of products after reduction with $LiAlH_4$		Absorption max. (nm) of TBA complexes		Chemical identity	
	I	II	I	II	I	II	I	II
β-Carotne	370	-	328	-	530	-	Retinal (370)	-
α-Carotene	370	245,360	328	310,325	530	520	Retinal	α-Retinal[1]
5,6-Epoxy-β-carotene	370	350	328	315	530	470	Retinal	5,6-Epoxy-retinal (352)
5,8-Epoxy-β-carotene	370	320,335	328	280	530	470	Retinal	5,8-Epoxy-retinal (317,331)
5,8,5',8'-Diepoxy-β-carotene	-	320,335	-	280	-	470	-	5,8-Epoxy-retinal
5,6-Epoxy-α-carotene	245,360	350	310,325	315	520	470	α-Retinal	5,6-Epoxy-retinal
5,8-Epoxy-α-carotene	245,360	320,335	310,325	280	520	470	α-Retinal	5,8-Epoxy-retinal
Cryptoxanthin	370	365	328	325	530	530	Retinal	3-Hydroxy-[2] retinal

..contd.

Table-1 (contd.)

Substrate used	Absorption max. (nm) in light petroleum of products formed		Absorption max. (nm) in light petroleum of products after reduction with $LiAlH_4$		Absorption max. (nm) of TBA complexes		Chemical identity	
	I	II	I	II	I	II	I	II
12'-Apo-β-carotenal	370	-	328	-	530	-	Retinal	-
10'-Apo-β-carotenal	370	-	328	-	530	-	Retinal	-
8'-Apo-β-carotenal	370	-	328	-	530	-	Retinal	-
12'-Apo-β-carotenol	370	-	328	-	530	-	Retinal	-
10'-Apo-β-carotenol	370	-	328	-	530	-	Retinal	-
8'-Apo-β-carotenol	370	-	328	-	530	-	Retinal	-
8'-Apo-β-carotenyl acetate	370	-	328	-	530	-	Retinal	-

( ) Absorption max.(nm) of authentic samples in light petroleum. 1. Absorption max. for α-retinal in ethanol (250,368 nm) reported by Robeson et al.(1955). 2. Absorption max. for 4-hydroxyretinal in ethanol (375 nm) reported by Barua and Nair (1962).

The compounds were incubated with the enzyme (from final purification step) under standard assay conditions and the products were isolated and processed as described in the text.

separated over 3 percent (v/w) water-deactivated neutral alumina (10 g) column (1x 12 cm). The column was first standardized using the authentic samples of substrates and their expected products. The method employed for the separation of substrates and their products is briefly described as follows:

1. β-Carotene from retinal: β-Carotene was eluted with light petroleum (25 ml) followed by the elution from the column using 5 percent (v/v) diethylether in light petroleum (25 ml).

2. α-Carotene from α-retinal and retinal: α-Carotene was eluted with light petroleum (25 ml) followed by the elution of the products retinal and α-retinal with 25 ml of 5 percent (v/v) diethylether in light petroleum.

3. 5,6-epoxy-β-carotene from retinal and 5,6-epoxy-retinal: 5,6-Epoxy-β-carotene was eluted first with 50 ml of light petroleum. Afterwards retinal was eluted with 5 percent (v/v) diethylether in light petroleum (25 ml) and 5,6-epoxyretinal with 8 percent (v/v) diethylether in light petroleum (25 ml).

4. 5,8-Epoxy-β-carotene from retinal and 5,8-epoxy-retinal: 5,8-Epoxy-β-carotene was eluted from alumina column with light petroleum (50 ml) followed by retinal with 25 ml of 5 percent (v/v) diethylether in light petroleum and 5,8-epoxyretinal with 25 ml of 10 percent (v/v) diethylether in light petroleum.

5. 5,8,5',8'-Diepoxy-β-carotene from 5,8-epoxyretinal: 5,8,5',8'-Diepoxy-β-carotene was eluted from alumina column with 2 percent (v/v) diethylether in light petroleum (25 ml). It was followed by elution of 5,8-epoxyretinal with 10 percent (v/v) diethylether in light petroleum.

6. 5,6-Epoxy-α-carotene from α-retinal and 5,6-epoxy-α-retinal: 5,6-Epoxy-α-carotene was eluted from the column with light petroleum (50 ml), α-retinal being eluted with 5 percent (v/v) diethylether in light petroleum (25 ml) and 5,6-epoxyretinal with 8 percent (v/v) diethylether in light petroleum (25 ml).

7. 5,8-Epoxy-α-carotene from α-retinal and 5,8-epoxyretinal: 5,8-Epoxy-α-carotene was eluted from the column with 50 ml of light petroleum. α-Retinal was eluted with 25 ml of 5 percent (v/v) diethylether in light petroleum and 5,8-epoxyretinal with 25 ml of 10 percent (v/v) diethyl ether in light petroleum.

8. Cryptoxanthin from retinal and 3-hydroxyretinal: 25 ml of 5 percent (v/v) diethylether in light petroleum were used to elute retinal. 3-Hydroxyretinal was eluted with 25 ml of 30 percent (v/v) diethylether in light petroleum and cryptoxanthin with 10 percent (v/v) acetone in light petroleum.

9. Anhydrolutein from 3-dehydroretinal and 3-hydroxyretinal: 3-Dehydroretinal was eluted with 25 ml of 5 percent (v/v) diethylether in light petroleum, 3-hydroxyretinal with 25 ml of 30 percent (v/v) diethylether in light petroleum and anhydrolutein with 10 percent (v/v) acetone in light petroleum (25 ml).

10. 8'-Apo-β-carotenyl acetate from retinal: 8'-Apo-β-carotenyl acetate was eluted from the column with 2 percent (v/v) diethylether in 25 ml of light petroleum followed by elution with 25 ml of 2 percent (v/v) diethylether in light petroleum. Retinal was obtained by elution with 25 ml of 5 percent (v/v) diethylether in light petroleum.

11. Separation of apo-β-carotenals from retinals: Retinal was separated carefully from apo-β-carotenals on alumina column. Elution of retinal was carried out first by using 25 ml of 5 percent (v/v) diethylether in light petroleum. Apo-β-carotenals were eluted with 8-10 percent (v/v) diethylether in light petroleum.

12. Apo-β-carotenols from retinal: 5 Percent (v/v) diethylether was used to elute retinal from the column and this was followed by elution with apo-β-carotenols, viz., 8'-, 10'- and 12'- with 30 percent (v/v) diethylether in light petroleum.

For quantitative estimation of products formed, the retinal and/or α-retinal fractions were taken, evaporated to

dryness and dissolved in known volume of ethanol and their concentration was estimated by TBA method excepting when 5,8,5',8'-diepoxy-β-carotene and anhydrolutein were used as substrates, in which case 5,8-epoxyretinal and 3-dehydro-retinal, respectively were separated and estimated by TBA method by reading the color at 470 and 550 nm. The total concentration of product formation (in terms of retinal) from various substrates in comparison to β-carotene was calculated by using appropriate corrections in each case.

RESULTS

Purification of Carotene 15,15'-dioxygenase from guinea pig and rabbit intestine.

Carotene 15,15'-dioxygenase enzyme from the intestinal mucosa of guinea pig was partially purified and the results of different purification steps are shown in Table-2. A purification of 38-fold over the crude enzyme activity was achieved with 277 percent recovery. Following a similar procedure the enzyme from rabbit was purified to about 30-fold with 200 percent recovery.

Table-2: Summary of purification of carotene 15,15'-dioxygenase from guinea pig intestine.

Step No.	Treatments	Total protein (mg)	Total activity	Specific activity	Fold purification
1	Crude	945.0	160.5	0.17	1.0
2	Protamin sulphate supernatant	765.0	251.7	0.33	1.9
3	Ammonium sulphate fractionation (25 - 55 percent)	571.0	224.4	0.39	2.3
4	Acetone precipitation (45 - 65 percent)	68.8	445.6	6.48	37.8

5.0 ml of enzyme protein from each step of purification was incubated with β-carotene under the standard assay conditions and the activity was determined by estimating the amount of retinal formed.

Disappearance of retinal by preparations at different steps of carotene 15,15'-dioxygenase purification.

To conclusively exclude the possibility of further utilization of retinal formed by the action of carotene 15, 15'-dioxygenase on carotenoids, the disappearance of retinal by the enzyme preparations taken from different steps of purification was followed. Results are shown in Table-3.

Table-3: Disappearance of retinal by preparations taken at different steps of purification of carotene 15,15'-dioxygenase from guinea pig intestine.

Treatment	Specific activity
Crude	20.0
Protamine sulphate supernatant	14.2
Ammonium sulfate fractionation (25-55 percent)	12.2
Acetone precipitation$^{\delta}$ (0-45 percent)	24.0
Acetone precipitation (45-65 percent)	0
Acetone precipitation (45-65 percent) from rabbit intestine	0

δ The fraction contained 1.3 mg of protein/ml in total volume of 30 ml.

Each preparation was incubated with retinal as described in the text and the disapperance of retinal was estimated.

It is evident from the Table, that there was disappearance of retinal in crude extract which decreased at subsequent steps of purification. It is interesting to note that the retinal disappearing activity was totally absent in the final preparation containing carotene 15,15'-dioxygenase. In the case of enzyme in rabbit intestinal mucosa, the same procedure yielded a final preparation which was devoid of retinal disappearing activity (Table-3).

Properties of partially purified carotene 15,15'-dioxygenase from guinea pig.

Effect of time: Time-course of carotene 15,15'-dioxygenase enzyme with β-carotene as substrate shows that the reaction is linear upto 3 hr. and reaches plateau stage within 4 hr. (Fig.1). In all studies, an incubation period of 3hrs. has been chosen for the assay of the enzyme activity.

Effect of pH: The influence of pH on the enzyme activity in the range of 6.0-10.0 is given in Fig.2. The buffers used for pH ranges are as follows:

0.1M potassium phosphate buffer for	pH 6.0-7.5
0.1M Tris-HCl buffer	pH 7.5-9.0
0.1M Carbonate-Bicarbonate buffer	pH 9.0-10.0

The optimum pH for the reaction is 8.5. The pH curve shows the bell-shaped nature. A decrease of 50 percent in enzyme activity is noticed at pH 7.5 and 9.2.

Substrate concentration: Fig.3 depicts the effect of varying amount of β-carotene on the enzyme activity. The activity exhibits a linear relationship upto the substrate concentration of 200 nmoles/reaction mixture and a plataeau stage is observed at higher concentrations. Therefore, 200 nmoles of substrate was employed for the enzyme assay. Km for β-carotene of 9.5×10^{-6}M and Vmax of 3.3 nmoles of retinal formed/mg protein/hr.(Fig.4).

Effect of protein concentration: The enzyme activity shows linearity upto the protein concentration of 25 mg/ reaction mixture.

Effect of sodium dodesyl sulfate (SDS): The effect of SDS from concentrations of 0-40 mg/incubation mixture on the enzyme activity was tested. Table-4 represents the influence of SDS. It is evident that increasing concentrations of detergent results in the inactivation of the enzyme.

Effect of reduced glutathione (GSH): Table-5 shows that activity increaes with increasing concentration of reduced glutathione. A concentration of 10 mg/reaction mixture of GSH

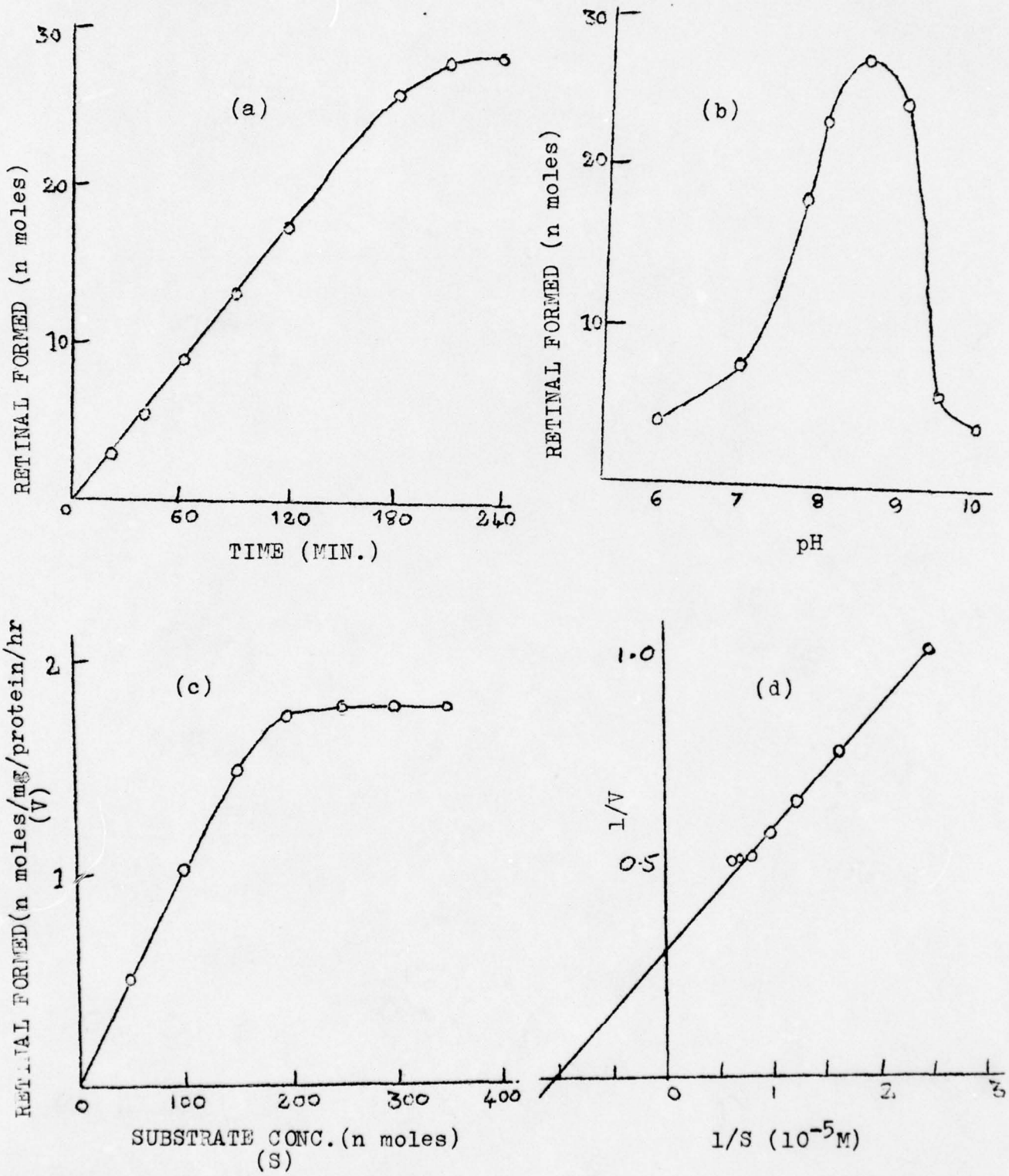


Fig.1: **(a) TIME COURSE**; (b) pH CURVE; (c) SBSTRATE SATURATION CURVE AND (d) LINEWEAVER-BURK PLOT

causing a 4.7-fold increase in the basal activity has been used throughout this study.

Effect of iron concentration: Iron, which is known to be the sole cofactor of dioxygenase enzymes, was tested in carotene 15,15'-dioxygenase isolated from guinea pig and the results are depicted in Table-5. Iron in the ferric state (Fe^{+++}) was found to be ineffective. Surprisingly, a stimulatory effect was exhibited by iron in Fe^{++} state. The enzyme was activated with supplementation of Fe^{++} and maximally at 1×10^{-3}M final concentration of Fe^{++}(approximately 6-fold). Concentrations higher than 1×10^{-3}M (final concentration) resulted in a decrease in enzyme activity. Enzyme activity was decreased by 60 percent when Fe^{++} concentration was raised by 1×10^{-3} to 4×10^{-3}M.

Table-4: Effect of sodium dodecyl sulfate (SDS) on the activity of carotene 15,15'-dioxygenase from guinea pig intestine.

Concentration (mg)	Specific activity	Percent activity
0	1.60	100
5	1.59	99
10	1.18	70
20	0.56	35
30	0.21	13
40	0.07	4

The reaction was carried out under standard assay conditions using 5.0 mg of enzyme protein/reaction mixture. The enzyme (from final purification step) was incubated with different concentrations of the detergent (SDS) prior to the addition of the substrate. The product (retinal) was separated from the substrate (β-carotene) by column chromatography over alumina and estimated by the method of TBA.

Table-5: Effect of reduced glutathione (GSH) on carotene 15, 15'-dioxygenase from guinea pig intestine.

Concentration (mg)	Specific activity	Percent activity
0	0.34	100
10	1.60	471
20	1.81	532
30	2.50	735
40	2.85	838

The final enzyme preparation was preincubated for 5 min with different concentrations of GSH and reaction was started by the addition of substrate (β-carotene). The activity was estimated under standard assay conditions using 5.0 mg of the enzyme protein/reaction mixture. The product (retinal) was separated from the substrate (β-carotene) by column chromatography over alumina and estimated by the method of TBA.

Table-6: Effect of iron (Fe^{++} and Fe^{+++}) on carotene 15,15'-dioxygenase from guinea pig intestine.

Supplement	Final concentration	Specific activity	Percent activity
Ferric sulfate	0	0.57	100
	0.1×10^{-3} M	0.55	96.8
	0.5×10^{-3} M	0.52	91.2
	1.0×10^{-3} M	0.56	98.3
Ferrous ammonium sulfate	0	0.57	100
	0.1×10^{-3} M	0.84	147
	0.5×10^{-3} M	2.02	354
	1.0×10^{-3} M	3.33	584
	2.0×10^{-3} M	2.86	502
	3.0×10^{-3} M	2.13	374
	4.0×10^{-3} M	1.35	237

The enzyme was preincubated with the metal ion for 5 min. before the addition of the substrate (β-carotene). Reaction was carried out under standard assay conditions using 5.0 mg of enzyme protein/reaction mixture. Retinal (product) was separated from the substrate by column chromatography over alumina and the concentration of retinal was estimated by the method of TBA.

Effect of iron chelating and sulfhydryl binding agents: The partially purified carotene 15,15'-dioxygenase is susceptible to iron chelating agents like α,α-dipyridyl and 1,10-phenanthroline at 1 x 10^{-3}M (final concentration) inhibt the enzyme to 60 percent and 75, respectively is shown in Table-7. Similarly, sulfhydryl binding agents, viz., p-chloromercuribenzoate (pCMB), iodoacetate and N-ethyl-maleimide inactivated the enzyme.

Stoichiometry of the reaction: The reaction catalyzed by enzyme from guinea pig intestinal mucosa is strictly stoichiometric. There is formation of 2 moles of retinal upon utilization of one mole of β-carotene when the concentrations of the enzyme protein is varied from 6.0-24.0 mg as shown in Table-8.

Comparison of carotene 15,15'-dioxygenase from guinea pig and rabbit: Enzyme isolated from the intestine of guinea pig and rabbit showed remarkable similarities with respect to time-course, iptimal substrate concentration, activation by Fe^{++} and GSH, inhibition by SDS, iron-chelating agents and sulphydryl binding agents. The only distinctive property observed was that of the optimal activity. Rabbit enzyme has an optimum pH of 7.8 while that of guinea pig has at 8.5.

Identification of reaction products from different ca rotenoid substrates: When different carotenoids were used as substrates for carotene 15,15'-dioxygenase, the products formed were isolated, purified and characterized. The chemical identities of retinal, α-retinal, 3-dehydroretinal, 5,6- and 5,8-epoxides of retinal and 3-hydroxyretinal were established by their identical absorption spectra with that of synthetic compounds in light petroleum. Fig.5 represents the absorption spectra of the products. The absorption maxima of isolated products, their corresponding alcohols as well as their TBA complexes are given Table-1.

Substrate specificity of carotene 15,15'-dioxygenase of guinea pig and rabbit: The substrate specificity of this enzyme

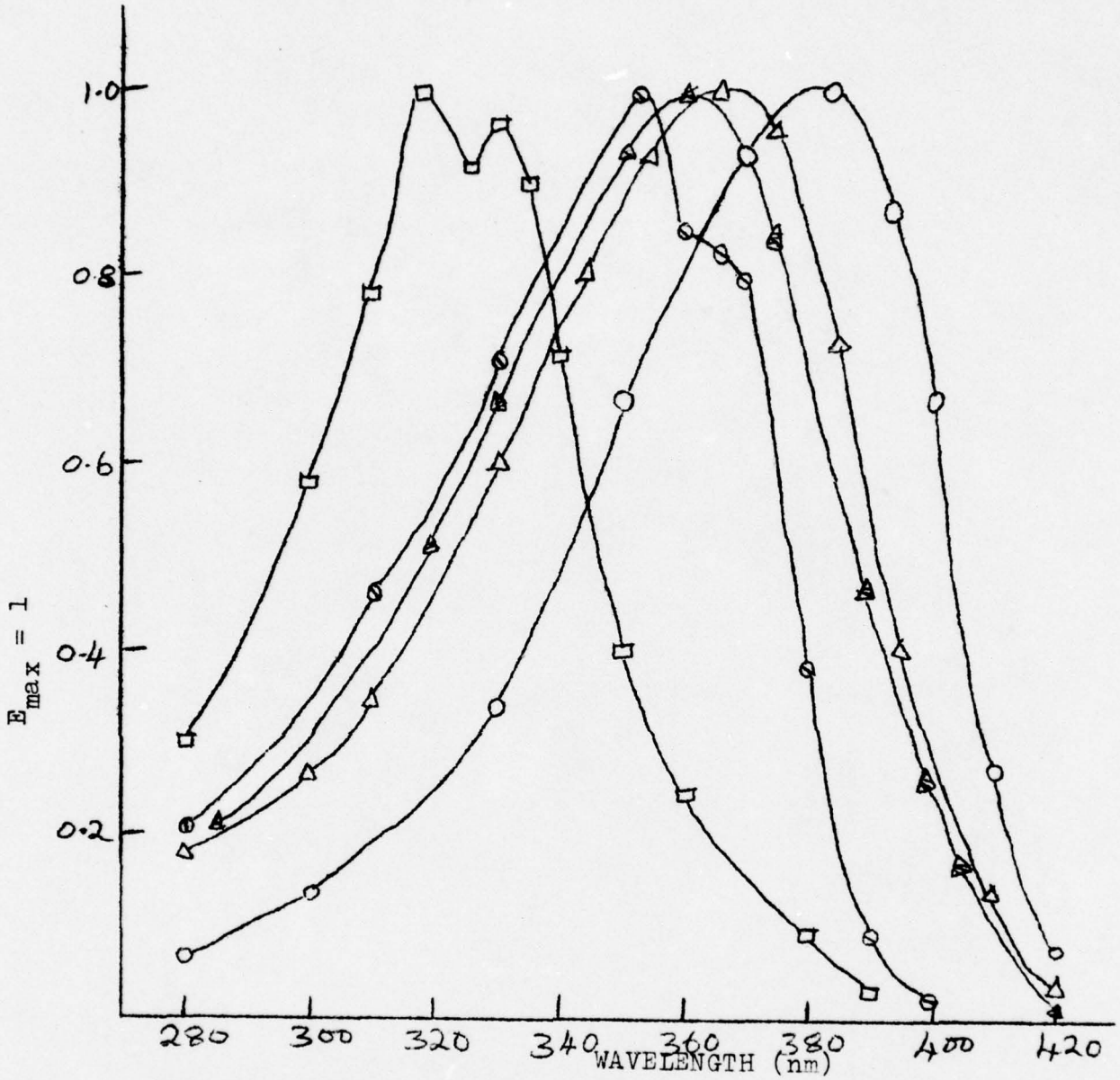


Fig.2: ABSORPTION SPECTRA OF REACTION PRODUCTS IN LIGHT PETROLEUM: (O) 3-DEHYDRORETINAL, (△) 3-HYDROXY-RETINAL AND RETINAL, (▲) α-Retinal, (⊘) 5,6-EPOXY-RETINAL AND (□) 5,8-EPOXYRETINAL.

is represented in Table-9. β-Carotene is found to be the most active substrate for the enzyme from both the sources. Carotenoids with modification in β-ionone ring such as α-carotene, 5,6- and 5,8-epoxides of α- and β-carotenes, 5,8, 5',8'-diepoxy-β-carotene, cryptoxanthin and anhydrolutein are poor substrates as compared to β-carotene, zeaxanthin and lutein are not cleaved.

The enzyme showed an interesting behaviour towards apocarotenoids. It can cleave apo-β-carotenoic acids. 5,6- and 5,8-Epoxides of 8'- and 12'-apo-β-carotenals, 5,6- and 5,8-epoxides of 8'-apo-β-carotenyl acetate, 3-acetoxy and 3-hydroxy-8'-apo-β-carotenals are not attacked by the enzyme. Amongst apo-β-carotenals and apo-β-carotenols, the enzyme shows maximum activity with 10'-apo-β-carotenal and 10'-apo-β-carotenol.

Table-7: Inhibition of carotene 15,15'-dioxygenase from guinea pig intestine by iron chelating and sulfhydryl binding agents.

Supplement	Final concentration	Percent inhibition
α,α'-Dipyridyl	10^{-3}M	60.0
1,10-Phenanthroline	,,	75.0
Iodoacetate	,,	65.4
N-Ethylmaleimide	,,	81.5
p-Chloromercuribenzoate (pCMB)	,,	85.2

The reaction was carried out under standard assay conditions. The enzyme (5.0 mg) (from last purification step) was incubated for 5 min. with different inhibitors prior to the addition of substrate (β-carotene). Retinal was separated from β-carotene by column chromatography and estimated by TBA method. The results are expressed as the percentage of activity inhibited as compared to control.

Table-8: Stoichiometry of carotene 15,15'-dioxygenase reaction from guinea pig intestine.

Enzyme protein/ reaction mixture (mg)	β-Carotene utilized	Retinal formed
6.0	10.5	20.5
9.0	16.4	33.5
12.0	24.0	46.0
15.0	30.0	59.5
24.0	40.0	78.0

β-Carotne (200 nmoles) was incubated with increasing concentrations of the enzyme (from last purification step) under standard assay conditions. β-Carotene and retinal were separated by column chromatography over alumina and estimated. Retinal was estimated by TBA method, whereas the concentration of β-carotene was estimated by recording optical density at 450 nm. ($E^{1\%}_{1\ cm}$ in light petroleum = 2500). The results are expressed as nmoles of substrate disappearance and product formation.

DISCUSSION

Carotene 15,15'-dioxygenase isolated from the intestinal mucosa of guinea pig and rabbit has been partially purified by 38- and 30-folds, respectively. The enzyme at the final purification step was found to be free of other enzyme activities responsible for further degradation of the product (retinal), which results in increase in total enzyme activity. A similar increase in carotene 15,15'-dioxygenase activity during purification from rabbit intestinal mucosa was also reported by Lakshmanan *et al.*(1972) which was attributed to the removal of endogenous inhibitor.

The reaction catalyzed by the guinea pig enzyme is strictly stoichiometric with two moles of retinal formation per mole of β-carotene disappearance. The guinea pig enzyme has Km for β-carotene of 9.5×10^{-6} M and Vmax of 3.3 nmoles of retinal formation per mg protein per hr. In comparison, the 27-fold purified hog intestinal enzyme has Km for

Table-9: Substrate specificity of carotene 15,15'-dioxygenase from guinea pig and rabbit intestinal mucosa.

Substrate	Guinea pig enzyme		Rabbit enzyme	
	Specific activity	Relative activity	Specific activity	Relative activity
β-Carotene	5.932	1.000	8.409	1.000
α-Carotene	4.225	0.716	4.667	0.555
5,6-Epoxy-β-carotene	1.920	0.324	2.123	0.252
5,8-Epoxy-β-carotene	1.111	0.187	0.911	0.108
5,8,5',8'-Diepoxy-β-carotene	0.101	0.017	0.122	0.015
5,6-Epoxy-α-carotene	1.462	0.247	1.810	0.215
5,8-Epoxy-α-carotene	0.548	0.092	0.712	0.085
Cryptoxanthin	0.222	0.037	0.217	0.026
Anhydrolutein	0.182	0.031	0.158	0.019
8'-Apo-β-carotenal	0.564	0.095	0.280	0.033
10'-Apo-β-carotenal	3.374	0.569	4.652	0.553
12'-Apo-β-carotenal	0.679	0.115	0.570	0.068
8'-Apo-β-carotenyl acetate	0.961	0.162	0.798	0.095
8'-Apo-β-carotenol	0.414	0.070	0.150	0.018
10'-Apo-β-carotenol	2.976	0.502	3.604	0.429
12'-Apo-β-carotenol	0.590	0.077	0.510	0.066

Different substrates were incubated with final preparation of enzyme from guinea pig and rabbit intestine (15.0 mg/reaction mixture) under the standard assay conditions. The activity was estimated by determining the amount of formation of products as described in the text.

β-carotene of 1.3 x 10^{-6}M and Vmax of 0.8 nmole of β-carotene cleaved/mg protein/hr. at 37°C (Fidge et al.,1969); the 2 to 4-fold purified rat intestinal enzyme has a Km of 3 x 10^{-6}M for β-carotene and a Vmax of 0.57 nmoles of β-carotene cleaved per mg protein/hr. at 37°C(Goodman et al.,1967).

The properties of carotene 15,15'-dioxygenase from both the animal species exhibited remarkable similarities being stimulated by GSH and Fe^{++} and inhibited by iron chelating and sulfhydryl binding agents. Rat dioxygenase is stimulated by SDS (Goodman et al.,1967) while SDS inhibits guinea pig, rabbit and hog enzyme. Activation of the rabbit and guinea pig enzymes at low concentrations of Fe^{++} and unusual pattern of activation at higher concentrations was observed.

The dioxygenase enzyme from both the species catalyze the scission of β-carotene at 15,15'-double bond to retinal. It cleaves other carotenoids like α-carotene to α-retinal and retinal; 5,6-epoxy-β-carotene to 5,6-epoxyretinal and retinal; 5,8-epoxy-β-carotene to 5,8-epoxyretinal and retinal; 5,8,5'8'-diepoxy-β-carotene to 5,8-epoxyretinal; 5,6-epoxy-α-carotene to 5,8-epoxyretinal and α-retinal; cryptoxanthin to 3-hydroxy-retinal and retinal; anhydrolutein to 3-hydroxyretinal and 3-dehydroretinal (Table-1). The enzyme is inactive against zeaxanthin and lutein. Though apo-β-carotenals and apo-β-carotenols are cleaved to retinal, yet the cleavage of apo-β-carotenoic acids could not be demonstrated. Modification of β-ionone ring in apo-β-carotenals either by epoxidation or by hydroxylation results in total loss of activity.

A change of β-ionone to α-ionone ring of β-carotene molecule lowers the activity to 71 and 55 percent in the cases of guinea pig and rabbit enzymes, respectively (Table-9). Any other modification at one of the β-ionone ring of α- and β-carotenes results in tremendous decrease in enzyme activity. Introduction of a hydroxyl group at one of the β-ionone ring of β-carotene molecule lowers the activity to 3-4 percent. Anhydrolutein is relatively less active than cryptoxanthin in both the species.

Amongst apo-β-carotenals and apo-β-carotenols tested, 10'-apo-β-carotenal and 10'-apo-β-carotenol are more readily cleaved than others. However, the cleavage of apo-β-carotenals and apo-β-carotenols is slower than that of β-carotene. Lakshmanan et al.(1968) observed that carotene 15,15'-dioxygenase from rabbit intestine can catalyze the cleavage of apo-β-carotenals at a much faster rate than that of β-carotene and the enzyme activity was in the decreasing order of 10' > 8' > 4' in apo-β-carotenols. This led to the suggestion that the increase in chain length of apo-β-carotenals results in decrease in their susceptibility to be cleaved by the enzyme. Recently, Lakshmanan et al.(1972) observed that carotene 15,15'-dioxygenase from rabbit and guinea pig intestine is respectively 12.5 and 5.4 times more active with 10'-apo-β-carotenol than β-carotene. However, our observations are not in agreement with these reports and no general correlation with respect to chain length and enzyme activity can be drawn in apo-β-carotenals. The high enzyme activity observed by Lakshmanan et al.(1968,1972) towards 10'-, 8'- and 4'-apo-β-carotenals and 10'-apo-β-carotenol may be due to two reasons: Firstly, the difference in the solubility of various substrates under experimental conditions and secondly, the enzyme reaction is very slow and to correlate the amount of substrate disappearance using crude enzyme preparations, with high degree of precision may cause experimental error. Our in vitro studies with apo-β-carotenals are supported by in vivo studies with these compounds and those reported by Glover (1960) where it was observed that the percentage of vitamin A formation from apo-β-carotenals in the rat is very low as compared to β-carotene.

It will be of interest here to discuss the in vitro results in the light of the in vivo studies. During the nutritional studies with 5,6-epoxy-β-carotene (Subbarayan et al.,1966),5,8-epoxy-β-carotene (Jacob John and Cama, 1972), cryptoxanthin (Jacob John et al.,1970), 5,6-epoxy-α-carotene (Kishore et al., 1970) and anhydrolutein (Savithry et al,1972)

an excentric cleavage was proposed to account for the biosynthesis of vitamin A, α-vitamin A or 3-dehydrovitamin A depending on the nature of the carotenoids. However, the detection of ring substituted retinal derivatives as reaction products in the present *in vitro* studies clearly establish that the ring substituted carotenoids are also cleaved at the 15,15'-double bond.

A comparison of our *in vivo* and *in vitro* studies with apo-β-carotenals indicates that these compounds are converted to vitamin A *in vivo* and are also cleaved to retinal enzymatically. On the other hand, epoxides of apo-β-carotenals and 3-hydroxy-8'-apo-β-carotenal which are not attacked at the 15,15'-double bond *in vivo* are also resistant to the attack by the enzyme. In *in vivo* studies with apo-β-carotenoic acids, it was found that these compounds have vitamin A activity as tested by feeding experiments and are converted to vitamin A, however, these compounds could not be cleaved to retinal by carotene 15,15'-dioxygenase. This anomaly may be due to very low enzymatic conversion of these compounds such that the trace amounts of retinal, if at all formed, could not be detected under experimental conditions.

These enzymatic studies conclusively show that the carotene 15,15'-dioxygenase enzyme can also cleave other carotenoids at 15,15'-double bond. It may be conceived that the enzyme has a very high specificity for the chemical environment of the β-ionone ring as well as for the polyene chain of β-carotene and the structural modification in the ring or in the chain affects the enzyme activity.

Effect of hypothermia on the metabolism of β-carotene.

Various physiological mechanisms ensue when the environmental temperature varies. The resistance of animals to cold environment is brought about by biochemical alterations and behavioural changes. Increase in basal metabolic rate contributes effectively for the heat production during cold acclimation. Metabolic rate in acute or chronic cold exposure

is probably mediated by increased production and utilization of thyroid hormone as evidenced by enlargement of thyroid and increased secretion of thyroid stimulating hormone (Bauman and Turner, 1967), cold acclimation has been found to depend on a marked rise in norephinephrine and epinephrine levels (LeBlanc and Nadeau, 1961).

It is well known that cold stress conditions bring about a host of changes in the metabolism of carbohydrate lipids, proteins, nucleic acids and minerals. Carotenoids are considered to be important dietary factors. Till today, a little is known regarding their metabolism under stress conditions. Sundaresan et al.(1967) reported that total retinol levels of liver were not affected by exposing rats to a cold environment. However, the increased utilization of retinol was indicated in cold exposed rats, when the utilization was expressed as a ratio of retinol utilization to weight gain of the animal. Further, Sundaresan and Therriault (1969) observed that retinal reductase level decreased in animals exposed to cold environment while retinal oxidase increased. They suggested that because rats are normally reared on a feed which consists of β-carotene as the precursor of retinol, the enzyme retinal reductase is initially adapted to β-carotene and favours storage of retinol. When the rats are placed on retinol-free diet, the rats tend to store less and increase utilization of retinol. Hence, the investigations were carried out to study the effect of cold on the absorption and utilization of β-carotene by the rat.

Materials and Methods

Materials: Materials are as described in Part-I.

Methods: Rats were made vitamin A-deficient as described in Part-I. They were divided into two groups: one group was exposed to low temperature (0-5°C) and the other group was maintained at room temperature (25-30°C). β-Carotene was fed orally to both the groups at the dosage of 0.5 mg/rat/day over a period of time depending on the experimental design.

The feeding of β-carotene was carried out upto the period of 3 weeks. The animals were killed at the intervals of 7,14 and 21 days. Livers were removed and vitamin A was extracted. Total vitamin A content and concentration of retinol and retinyl esters were estimated by employing 3-point corrections (Cama et al.,1951).

Estimation of retinol and retinyl ester ratio: The crude extract in light petroleum was loaded on to 5 percent (w/w) water deactivated alumina column. Retinyl esters were eluted with light petroleum, the strongly adsorbed retinol being eluted later with 30 percent ether in petrol. They were estimated by measuring the absorbance at 328 and 325 nm, respectively in ethanol.

RESULTS AND DISCUSSION

Table-1 describes the liver storage of vitamin A after feeding of β-carotene orally.

Table-1: Liver storage of vitamin A after oral feeding of 0.5 mg β-carotene/rat/day.

No. of days	Total amount of vitamin A/rat in µg	
	Control	Experimental
7 (5)	119 ± 5.4	102 ± 8.2
14 (4)	122 ± 7.8	106 ± 4.5
21 (4)	129 ± 6.4	67 ± 8.8

Figures in parenthesis indicate the total number of animals taken for experiment.

It is evident from the Table-1 that the relative amount of vitamin A in the livers of rats exposed to cold is lower than that corresponding control animals indicating that probably the utilization of vitamin A in cold-exposed animals may be higher than the animals kept at room temperature. Table-2 shows the ratio of retinol to retinyl esters on feeding of β-carotne.

Table-2: Ratio of retinol to retinyl esters in cold-exposed animals after feeding of 0.5 mg β-carotene.

No. of days	Retinol/Retinyl ester ratio	
	Control	Experimental
7 (5)	0.099 ± 0.02	0.133 ± 0.005
14 (4)	0.089 ± 0.005	0.114 ± 0.002
21 (4)	0.099 ± 0.003	0.119 ± 0.004

Figures in parenthesis indicate the number of animals.

The ratio of retinol to retinyl ester goes up in the livers of rats exposed to low temperature. This may be taken as indication that more retinyl esters are hydrolyzed to retinol in the liver which can be utilized by the animal. The higher ratio of retinol to retinyl esters indicates that the enzyme which converts retinol to retinal, i.e., retinal reductase may be the rate limiting enzyme under cold exposure. The observation is in line with the enzymatic studies reported earlier (Sundaresan and Therriault, 1969) that activity of retinal reductase in cold exposed animals was lowered. It appears that more of retinol may be circulated in the plasma bound to specific transporting protein and transported to the tissue where it is required.

It is known that thyroid hormones enhance considerably the absorption of carotenoids from the gut. In turn this results in increased storage of vitamin A in the liver (Cama and Goodwin, 1949), indicating that cleavage of carotenoids to vitamin A in the intestine is probably influenced. However, from the present studies no such conclusion on the utilization of carotenoids by the cold exposed animals can be drawn.

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P A R T - III

Effect of Hypobaric Hypoxia Conditions on Lysosomes.

Living organisms interact not only with one another but also with a fluctuating environment. However, dynamic internal constancy in spite of external change is the vital quality of an organism. Hence, when homeostasis of an organism is challenged by environment or nutritional alterations organism adopts in such a manner to increase its survival efficiency in its particular habitat. During biochemical adaptation under stress conditions one or many vulnerable sites in metabolic control hierarchy may be altered. At the intracellular level lysosomal system appears to be a vulnerable site because of the fact that it is drastically perturbed by fluctuations in intra- and extracellular environment. The array of constituent acid hydrolases in lysosomes exhibits a voracious capacity to hydrolyze virtually all kinds of macromolecules. Hence, in the present investigation, the attention is focussed on the studies on the effect of high altitude hypoxia on lysosomes and the possible repercussions on the role of lysosomes due to impact of stress stimulus.

When animals are exposed to high altitude conditions they experience simultaneously two types stress conditions, viz., low oxygen tension (hypoxia) and low temperature (hypothermia) apart from low pressure (hypobaria). Since, simulation of high altitude conditions leading to hypobaric hypoxia modifies the metabolism of homeostatic mammals it is of interest to study the influence of this stress condition on lysosomes.

Materials and Methods

p-Nitrocatechol, p-nitrocatechol sulfate dipotassium salt, p-nitrophenyl phosphate disodium salt, Torulla yeast RNA, phenolphthalein β-glucuronic acid sodium salt, merthiolate and Triton X-100 were purchased from Sigma Chemicals, St. Louis, Mo.,U.S.A. Phenolphthalein was obtained from E.Merck,AG,

Darmstadt (west Germany). Hemoglobin was prepared after Anson (1939). Uranyl acetate was obtained from Koch-Light Chemicals. All the other chemicals were of Analar grade. The solvents employed were distilled and used.

Methods.

Simulation of high altitude conditions: Female albino rats weighing 120 g were obtained from the Institute colony. They were fed on a rat pellet diet (Hindustan Lever Ltd., Bombay). Water was given ad libitum. Rats were exposed to half atmospheric pressure corresponding to the altitude of about 6100 m in a specially designed decompression chamber. The decompression of air was achieved by constant evacuation using a vacuum pump. At the same time, a leak was maintained. Thus, a free flow of air was maintained and the pressure at the half atmosphere. This pressure was obtained within 3 min after the start of evacuation and maintained constant value. Temperature can be regulated in the chamber. Throughout the experiment temperature was kept at 28°C to nullify the effect due to hypothermia observed at high altitude. Essentially, the animals experienced hypobaric hypoxia. Animals were exposed for different time intervals. The control animals were normobaric and kept at room temperature. For each time interval six animals were used.

Animals were killed by decapitation. Brain and liver were excised and kept in beakers placed in ice.

Tissue homogenization and preparation of lysosomal-rich fractions: Liver was minced well and homogenized in 0.25M sucrose solution (1:8 w/v) using Potter-Elvehjem homogenizer. The nuclear and mitochondrial fractions were removed by centrifuging the homogenate at 1000xg and 6500xg for 10 min each in a Sorvall RC-2B refrigerated centrifuge. For some experiments these pellets were washed once. The postmitochondrial supernatant, thus obtained, was centrifuged at 17,000xg for 30 min. The pellet was designated as lysosomal-rich fraction. It was washed in some experiments

once by resuspension in 0.25M sucrose and pelletting it at 12,000xg for 30 min. The activities of lysosomal enzymes, viz., acid phosphatase, aryl sulfatase, acid ribonuclease (acid RNase), cathepsin D and β-glucuronidase were assayed in homogenate nuclear mitochondrial and lysosomal-rich fractions in the presence of Triton X-100(1mg/0.1 ml). The 12,000xg supernatant was spun at 100,000xg for 60 min in a Spinco L model Beckman Ultracentrifuge. The supernatant, thus obtained, was assayed for the non-sedimentable free activity of these enzymes.

Brain was homogenized in 0.25M sucrose using a Potter-Elvehjem homogenizer. The total activity of the enzymes was estimated in the homogenate in the presence of Triton X-100 (1 mg/0.1 ml). The homogenate was centrifuged at 100,000xg for 60 min in a L-50 rotor in a Spinco L-model Ultracentrifuge. The supernatant, thus, obtained, was estimated for lysosomal enzymes and the activity was designated as free activity.

Assay of lysosomal enzymes

Acid phosphatase: Acid phosphatase was estimated using p-nitrophenyl phosphate as substrate by the method of Lowry et al.(1954). The assay system consisted of 0.2 ml of p-nitrophenyl phosphate (0.008M), 1.0 ml of 0.05M acetate buffer, pH 5.0 and appropriate protein concentrations in a 2 ml incubation mixture. Triton X-100 (1 mg/0.1 ml) was included in the cases of assay of total activity in homogenates of liver and brain and bound activity in liver lysosomal-rich, mitochondrial, nuclear and microsomal fractions. Triton X-100 was eliminated in the assay of free activity in the supernatant. The reaction mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 3 ml of 0.2M glycine-NaOH buffer, pH 10. A reagent blank was run in parallel. The intensity of the yellow colour was measured in Klett-Summerson Colorimeter using 42 filter. The calibration curve was constructed with p-nitrophenol as standard. In the case of total activity in liver homogenate,

activity was expressed as µmole of p-nitrophenol released/min while specific activities in bound and free forms as nmole of p-nitrophenol released/mg protein.

Aryl sulfatase: Activity was determined employing p-nitrocatechol sulfate as substrate, according to the method of Roy (1953). The incubation mixture of 2 ml contained 0.2 ml of 0.066M p-nitrocatechol sulfate, 1 ml of 0.5M acetate buffer, pH 5.7 and suitable protein concentrations. Triton X-100 (1mg/0.1 ml) was included for the assay of enzyme in all the fractions except in supernatant. The reaction was terminated after 30 min at 37°C, by adding 2 ml of 1N NaOH. The intensity of red colour was read at 515 nm in a Beckman DU-2 Spectrophotometer. A blank was run along with samples. A standard curve was plotted using p-nitrocatechol as standard. Total activity in liver was expressed as µmole of p-nitrocatechol liberated /min and specific activities in the bound and free forms as nmoles of p-nitrocatechol liberated/mg protein.

β-Glucuronidase: This enzyme is assayed according to the method of Gianetto and deDuve (1955) using phenolphthalein-β-D-glucuronide as substrate. The assay system consisted of 2 ml, comprising 0.5 ml of 0.0013M phenolphthalein β-D-glucuronide, 1 ml of 0.05M acetate buffer, pH 5.2 and appropriate protein concentrations. Triton X-100 (1 mg/0.1 ml) was used in all fractions except in the supernatant. The assay was carried out at 37°C for 20 min. The reaction was stopped by adding 0.2M glycine-NaOH buffer, pH 10.7. A reagent blank was estimated in parallel. The intensity of the pink colour was read in Klett-Summerson Colorimeter using 54 filter. Total activity in liver homogenate was expressed as µmoles of phenolphthalein released /min and specific activities in other fractions as nmoles of phenolphthalein released/mg protein.

Cathepsin D: The assay was carried out employing hemoglobin as substrate. Four ml of incubation mixture contained 2.5 ml of acid denatured hemoglobin (2.5 percent w/v) in buffer, 0.5 ml of 0.27M acetate buffer, pH 3.8 and suitable

protein concentrations. Triton X-100 (1 mg/0.1 ml) was included in all the fractions excepting the supernatant. The assay system was incubated for 30 min at 37°C and terminated by adding 5 ml of 5 percent trichloroacetic acid (TCA). Substrate and enzyme blanks were run in parallel. After precipitation with TCA it was kept in cold atleast for one hr. It was then centrifuged at 2,000 r.p.m. in cold. An aliquot of the supernatant was assayed for the released tyrosine using Folin's reagent, using tyrosine as standard. Total activity was expressed as μmoles of tyrosine equivalents released/min and specific activities as nmoles of tyrosine equivalents released/mg protein.

Acid ribonuclease: It was estimated by measuring the absorption of the released nucleotides at 260 nm from Torulla yeast RNA. Assay system of 3 ml consisted of 3 mg of purified RNA, 1.0 ml of 0.1M acetate buffer, pH 5 and appropriate protein concentrations. Triton X-100 (1 mg/0.1 ml) was employed in all fractions except in the supernatant. The reaction mixture was incubated at 37°C or 25°C for 30 min. The reaction was stopped by adding 0.5 ml of 0.75 percent uranyl acetate in 25 percent perchloric acid. The precipitate was allowed to settle in cold. It was then centrifuged at 2,000 r.p.m. in cold. An aliquot of supernatant was diluted suitably (using 0.1 ml to 3 ml dilution). The absorbance of the diluted samples was read at 260 nm. A substrate blank was included. The calibration curve was constructed using adenine as standard. Total activity was expressed as μmoles of adenine equivalents released/min and specific activities as nmoles of adenine equivalents released/mg protein.

Structural latency and extent of fragility of lysosomes

The activity present in supernatant was designated as free activity (F) and the activity that had to be released by Triton X-100 was referred to as bound activity. In the case of liver lysosomal-rich fraction was estimated for bound activity (B). Activity assayed in homogenate in the presence of Triton X-100 was called as total activity (T) while

calculating the percent free activity, the total activity was computed adding free and bound activities (B+F). This mode of computation was adopted to account for the possibility that the B+F was not equivalent to T. Moreover, during incubation at 37°C, the enzymes may be released from bound form to free and hence percent free activity may not reflect the true estimate. Further, the distribution of the enzymes in liver lysosomal-rich fractions of experimental animals did not differ very much from that of controls. Hence, in the case of liver, the percent free activity was always computed on the basis of (F/B+F) x 100.

Usually lysosomal enzymes exhibit structural latency i.e., they do not act on substrates without being released from the bound state by the action of disrupting agents. Hence, the extent of free activity, i.e., (F/B+F) x 100 in the case of liver and (F/T) x 100 in brain was considered to connote the extent of loss of structural latency or fragility of lysosomes.

Study of the distribution of enzymes in liver: The distribution of lysosomal enzymes in various fractions was determined. Different fractions of rat liver were prepared according to the method of Hogebroom et al.(1948) using 0.25M sucrose as homogenizing medium. The lysosomal enzymes viz., acid phosphatase, aryl sulfatase, acid RNase, β-glucuronidase and cathepsin D were estimated in homogenate, nuclear, mitochondrial lysosomal-rich microsomal and post-microsomal preparations. Except for supernatant fraction Triton X-100 (1 mg/0.1 ml) was used to release the sedimented enzyme activities.

Effect of osmotic stress on the isolated lysosomal-rich fractions: To investigate the aggravating influence of hypotonic sucrose solution on lytic susceptibility of hepatic lysosomal-rich fractions, the lysosomal-rich pellets were washed once and suspended in 0.25M sucrose. They were diluted appropriately with distilled water to give varying sucrose concentrations viz., 0.2, 0.15, 0.1 and 0.05M in

different Sorvall tubes. These tubes were preincubated for 30 min at 0°C (Deter and deDuve, 1967). After 30 min, these tubes were centrifuged at 17,000xg for 30 min. The enzyme activities leached out to the supernatant and associated with sediment were measured. Sedimented activities were estimated after treatment with Triton X-100 (1 mg/0.1 ml). In the present investigations acid phosphatase, aryl sulfatase, and acid RNase were determined in these fractions. The percent activity retained in the pellet was computed on the basis that the activities obtained by treatment with Triton X-100 (1 mg/0.1 ml), of lysosomal-rich fractions represented the total (100 percent). Thus, the percent free activity liberated to supernatant or percent activity retained in the sediment was considered to denote the injurious influence of osmotic stress conditions on the lytic susceptibility of lysosomal preparations.

Autolytic influence of temperature on the isolated liver lysosomal-rich fractions: The influence of incubation at 37°C on the augmented release of lysosomal enzymes from isolated hepatic lysosomal-rich/fractions were incubated at 37°C for different time intervals, viz., 0, 15, 30, 45, 60, 75 and 90 min. After the respective time intervals, the tubes were chilled and centrifuged at 17,000xg for 30 min. The supernatant was assayed for the released enzyme activities and the sediment for the retained acid phosphatase and aryl sulfatase were estimated in both. As above, the percent free activity released and the percent activity retained were computed. /fractions was evaluated as follows:these

Influence of ionic strength on the solubility of acid phosphatase: The effect of exposure to varying ionic strength on the augmented release of acid phosphatase was estimated by incubating isolated hepatic lysosomal-rich fractions with varying buffer concentrations for 1 hr. at 0°C. After the pre-incubation, the tubes were centrifuged at 17,000xg for 30 min. The activities retained in the pellet and the released into the non-sedimentable portions

were determined as described earlier.

Permeability of lysosomal membrane to p-nitrophenyl phosphate: Isolated liver lysosomal-rich fractions were incubated in test tubes with different concentrations of p-nitrophenyl phosphate for 1 hr. at 0°C. A blank assay was carried out by incubating corresponding lysosomal-rich fractions without p-nitrophenyl phosphate. After 1 hr., they were incubated at 37°C for 15 min in the absence of Triton X-100. Acid phosphatase liberated into supernatant was assayed as detailed earlier.

RESULTS

Effect of high altitude hypoxia on structural latency of lysosomal enzymes:

Fig.-I shows the influence of exposure of rats to hypobaric hypoxia on percent free activities of liver lysosomal enzymes viz., acid phosphatase, β-glucuronidase and acid RNase for different time intervals. It is evident that exposure to high altitude hypoxic conditions led to loss of structural latency of liver lysosomes. Striking feature was that exposure to simulated high altitude even for 1 hr. evoked the loss of structural latency. After 23 hr. of exposure the maximum percent free activities of lysosomal enzymes after 24 and 36 hr. of exposure showed that these decreased as compared to the values observed after 23 hr. of exposure. However, these values were still at an elevated level when compared to unexposed control animals. A similar picture was obtained in the case of brain lysosomal enzymes.

Tables 1 to 5 demonstrate the influence of hypobaric hypoxia on the total activities of the enzymes in whole and per gram liver and total specific and percent free activities of acid phosphatase, acid RNase, β-glucuronidase, cathepsin D and aryl sulfatase for different time intervals viz., 0.1,4, 12,24 and 36 hr. of exposure. Total activities of β-glucuronidase, acid phosphatase and cathepsin D increased. This may signify the increased synthesis.

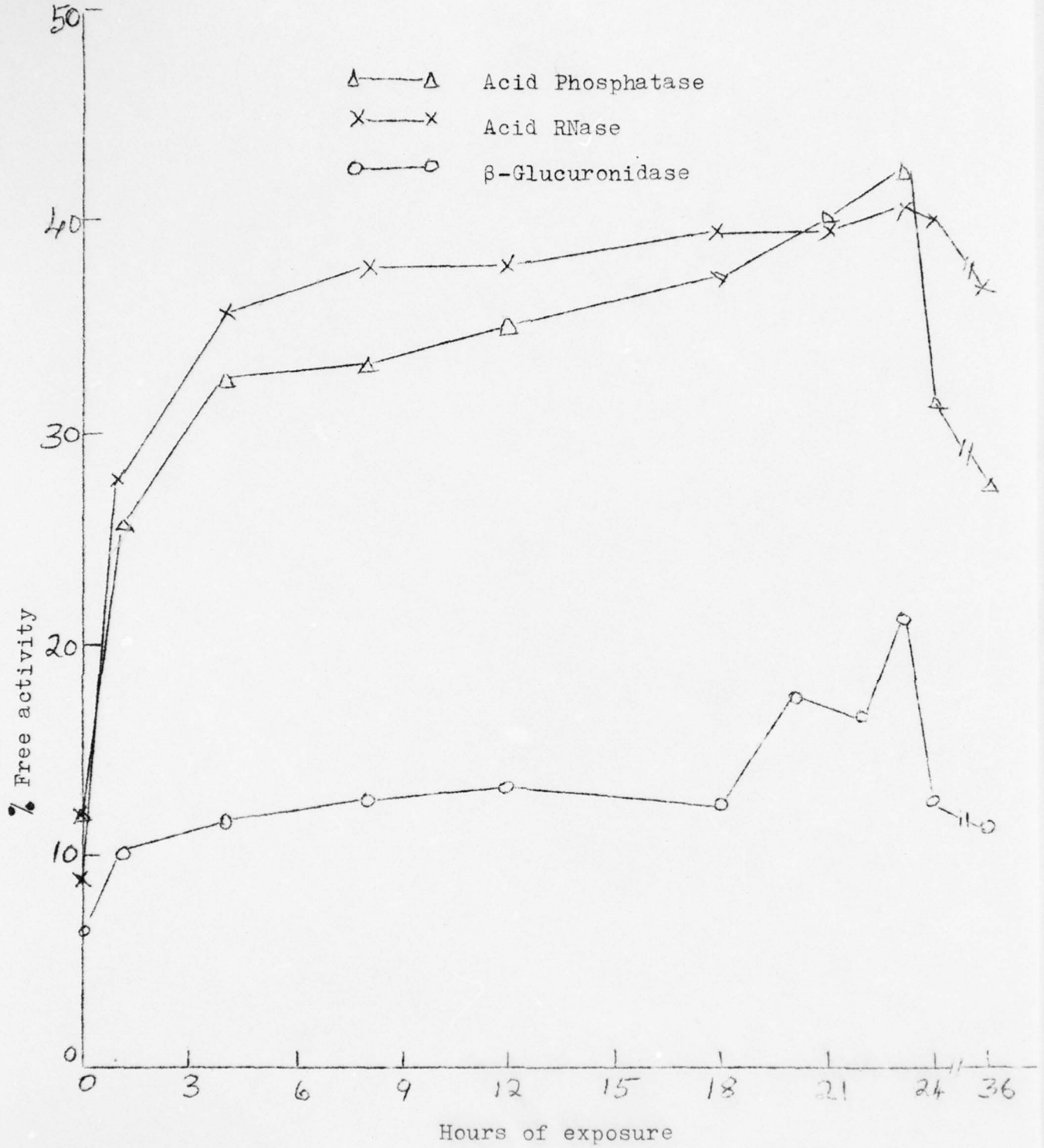


Fig.1: EFFECT OF EXPOSURE TO HYPOBARIC HYPOXIA ON THE FREE ACTIVITY OF LYSOSOMAL ENZYMES.

Table-1: Effect of high altitude hypoxia on structural latency of liver lysosomal acid phosphatase

Hours of exposure	Total activity[δ]			Total specific activity[δδ]		Percent free activity[δδδ]	
	in whole liver	per gm liver					
0	15.8±1.2	4.2±0.07		289±7.6		12.9±1.1	
1	25.4±2.4	6.7±0.25	P < 0.001	385±18.8	P < 0.001	25.5±1.4	P < 0.001
4	30.2±1.6	7.5±0.34	P < 0.001	394±16.1	P < 0.001	32.5±1.8	P < 0.001
12	32.4±0.8	8.2±0.42	P < 0.001	361±16.6	P < 0.001	35.4±2.0	P < 0.001
24	35.2±0.9	8.5±0.39	P < 0.001	629±12.5	P < 0.001	31.5±1.1	P < 0.001
36	32.6±0.2	8.0±0.25	P < 0.001	531±18.0	P < 0.001	28.0±7.2	P < 0.001

δ Total activity is expressed as μmoles of p-nitrophenol released per min.

δδ Total specific activity is expressed as nmoles of p-nitrophenol released per mg protein per incubation time.

δδδ Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

P > 0.05 Not significant; P < 0.05 Highly significant. The values are statistically evaluated by comparing with that of 0 hr exposure as control.

Table-2: Effect of high altitude hypoxia on structural latency of liver lysosomal acid ribonuclease.

Hours of exposure	Total activity$^{\delta}$			Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
	in whole liver	Per gm liver					
0	9.17±0.14	1.97±0.04		397±9.4		8.5±0.8	
1	10.74±0.83	2.39±0.18	P < 0.001	474±10.3	P < 0.001	27.5±1.7	P < 0.001
4	14.3±0.24	3.41±0.32	P < 0.001	525±15.2	P < 0.001	35.2±2.9	P < 0.001
12	11.79±0.48	3.13±0.19	P < 0.001	504±13.0	P < 0.001	37.7±1.8	P < 0.001
24	14.9 ±0.64	3.27±0.06	P < 0.001	539±14.3	P < 0.001	37.5±1.2	P < 0.001
36	15.3 ±0.37	3.41±0.23	P < 0.001	507±9.4	P < 0.001	36.7±2.4	P < 0.001

δ - Total activity is expressed as µmoles of adenine equivalents released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of adenine equivalents released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

The values are statistically evaluated by comparing with that of 0 hr exposure as control.

P > 0.05 Not significant; P < 0.001 Highly significant.

Table-3: Effect of high altitude hypoxia on structural latency of liver lysosomal cathepsin D

Hours of exposure	Total activity$^{\delta}$			Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
	in whole liver	per gm liver					
0	4.9±0.06	0.89±0.04		149±6.7		8.9±0.7	
1	6.1±0.31	0.95±0.12	P 7 0.05	172±9.4	P ⟨ 0.05	24.1±0.1	P ⟨ 0.001
4	10.1±1.20	1.40±0.34	P ⟨ 0.05	221±8.0	P ⟨ 0.001	27.4±2.4	P ⟨ 0.001
12	10.8±1.60	1.35±0.26	P ⟨ 0.05	215±11.6	P ⟨ 0.001	32.4±1.0	P ⟨ 0.001
24	12.3±1.90	1.61±0.39	P ⟨ 0.05	197±5.8	P ⟨ 0.001	35.5±0.6	P ⟨ 0.001
36	10.9±2.40	1.93±0.42	P ⟨ 0.05	188±4.5	P ⟨ 0.001	23.1±3.2	P ⟨ 0.001

δ - Total activity is expressed as µmoles of tyrosine equivalents released per min.

$\delta\delta$ - Total specific a ctivity is expressed as nmoles of tyrosine equivalents released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

The values are statistically evaluated by comparing with that of 0 hr exposure as control.

P 7 0.05 Not significa nt; P ⟨ 0.05 Highly significant.

Table-4: Effect of high a ltitude hypoxia on structural latency of liver lysosomal aryl sulfatase

Hours of exposure	Total activity$^{\delta}$ in whole liver	Total activity$^{\delta}$ per gm liver		Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
0	10.8±1.19	2.85±0.17		220±5.3		3.7±0.2	
1	17.4±1.35	4.22±0.39	P < 0.01	252±10.5	P < 0.05	12.5±0.8	P < 0.001
4	20.2±2.43	4.93±0.59	P < 0.01	278±7.5	P < 0.001	14.3±0.7	P < 0.001
12	21.4±2.17	4.97±0.28	P < 0.001	243±7.1	P < 0.05	11.7±0.9	P < 0.001
24	23.6±2.35	5.04±0.61	P < 0.01	305±11.5	P < 0.001	10.6±0.7	P < 0.001
36	20.7±1.39	4.72±0.27	P < 0.001	372±13.1	P < 0.001	8.4±0.4	P < 0.001

δ - Total activity is expressed as µmoles of p-nitrocatechol released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of p-nitrocatechol released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations

The values are statistically evaluated by comparing with that of 0 hr exposure as control.

P 7 0.05 Not significant; P < 0.05 Highly significant.

Table-5: Effect of high altitude hypoxia on structural latency of liver lysosomal β-glucuronidase.

Hours of exposure	Total activity$^{\delta}$ in whole liver	Total activity$^{\delta}$ per gm liver		Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
0	4.62±0.35	1.14±0.11		84±3.1		6.4±0.9	
1	7.25±0.61	2.28±0.61	P < .001	223±16.6	P < 0.001	10.1±0.6	P < 0.001
4	10.36±1.62	2.56±0.27	P < .001	256±15.6	P < 0.001	11.5±0.4	P < 0.001
12	15.21±0.69	3.23±0.38	P < .001	333±10.7	P < 0.001	12.6±1.6	P < 0.001
24	16.17±1.31	3.41±0.29	P < .001	281±7.6	P < 0.001	12.5±1.3	P < 0.001
36	14.23±1.53	2.92±0.33	P < .001	253±8.9	P < 0.001	11.5±1.4	P < 0.001

δ - Total activity is expressed as μmoles of phenolphthalein released per min.

δδ - Total specific activity is expressed as nmoles of phenolphthalein released per mg protein per incubation time.

δδδ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

The values are statistically evaluated by comparing with that of 0 hr exposure as control.

Experimental details are described in text.

P 7 0.05 Not significant; P < 0.05 Highly significant.

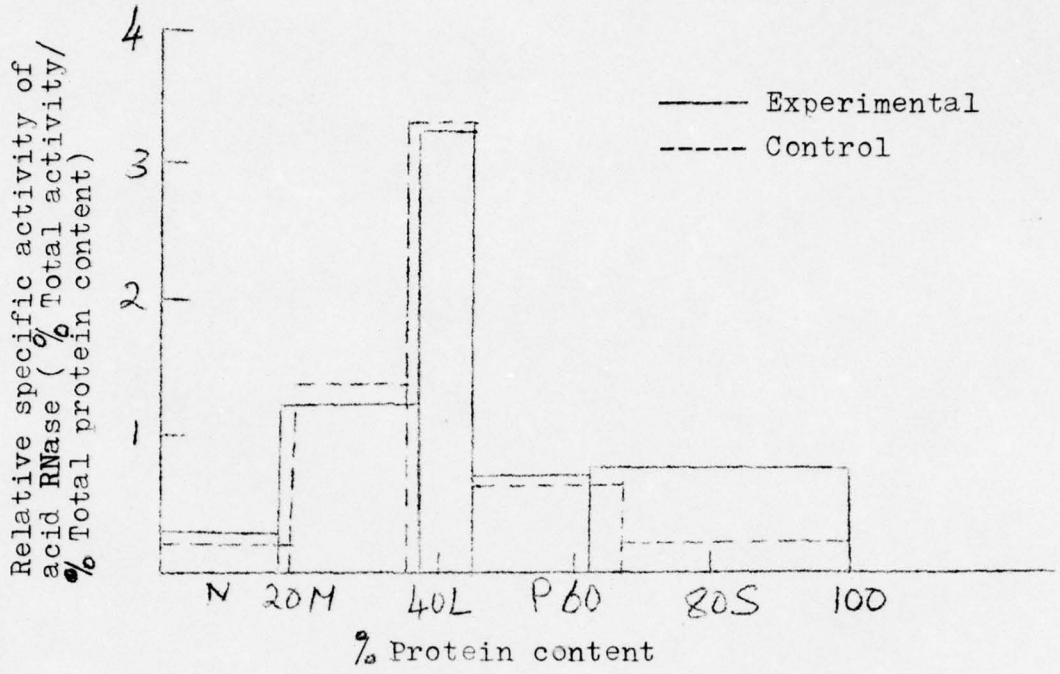
Tables 6 to 9 present the total activities in brain and percent free activities of acid phosphatase, acid RNase. aryl sulfatase and cathepsin D for different time intervals of exposure viz., 0, 1, 4, 12, 24 and 36 hr. A similar pattern was observed as in liver.

Increased percent free activities were considered to indicate the loss of structural latency of lysosomes. Such enhanced percent free activities of these enzymes may be due to increased fragility of lysosomal preparations obtained from exposed animals, to the homogenization technique.

Distribution of lysosomal enzymes among different fractions: Distributions of acid phosphatase, acid RNase, aryl sulfatase and cathepsin D were studied in different fractions viz., homogenate, nuclear mitochondrial, lysosomal-rich, microsomal and post microsomal supernatant. Fig.II shows the distribution of acid phosphatase and acid RNase in different fractions. It is evident that there was no demonstrable variation in the distribution of enzymes. Aryl sulfatase and cathepsin D exhibited a similar pattern.

Effect of osmotic stress conditions on lytic susceptibility of liver lysosomal-rich fractions: Increased free activities of the enzymes in animals exposed to hypobaric hypoxic conditions indicated the enhanced fragility of lysosomes. Hence, the effect of osmotic stress conditions was studied on the isolated lysosomal-rich fractions obtained from animals exposed to hypobaric hypoxic conditions for 4 hr. Figs.III to V reveal that exposure to hypotonic sucrose solutions accentuated the liberation of/acid RNase from lysosomal-rich fractions from animals exposed to hypobaric hypoxic conditions into the supernatant. The increase in percent free activity released to particulate-free supernatant or the decrease in percent retention of enzyme on exposure to osmotic stress conditions was more pronounced for experimental than that of control animals. The released enzymes may stem either from intact lysosomes or from the damaged lysosomes adsorbed to the intact ones.

/acid phosphatase, aryl sulfatase and



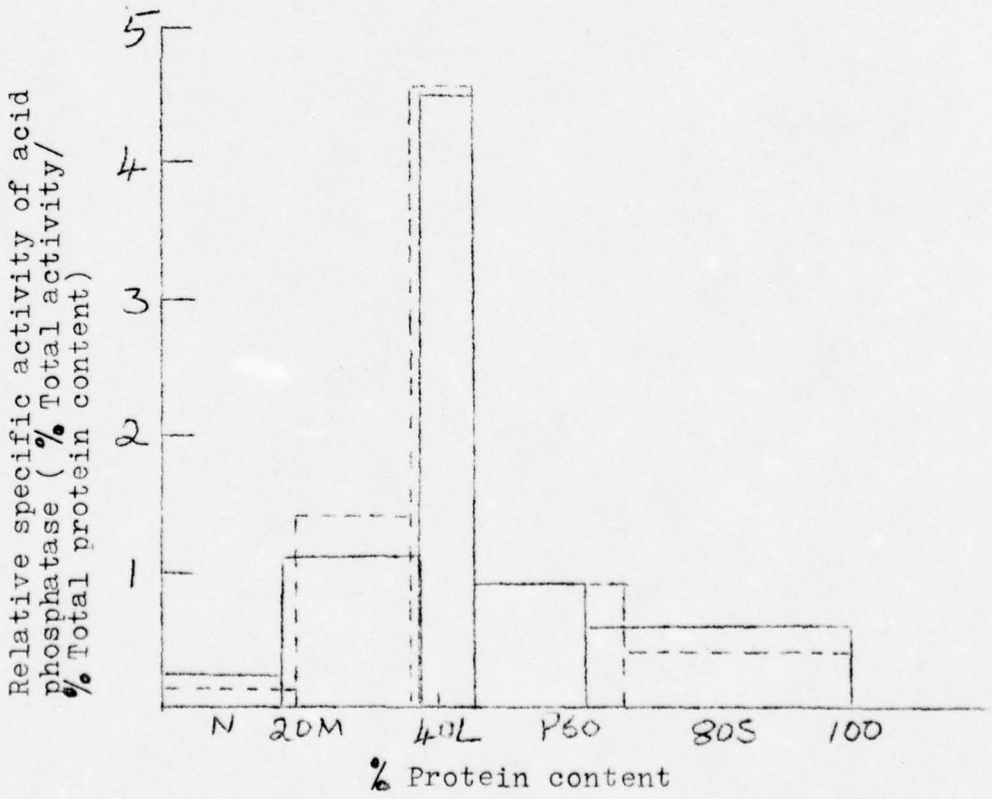


Fig.2: DISTRIBUTION OF ACID PHOSPHATASE AND ACID RNase IN DIFFERENT FRACTIONS OBTAINED FROM LIVER-INFLUENCE OF HYPOBARIA.

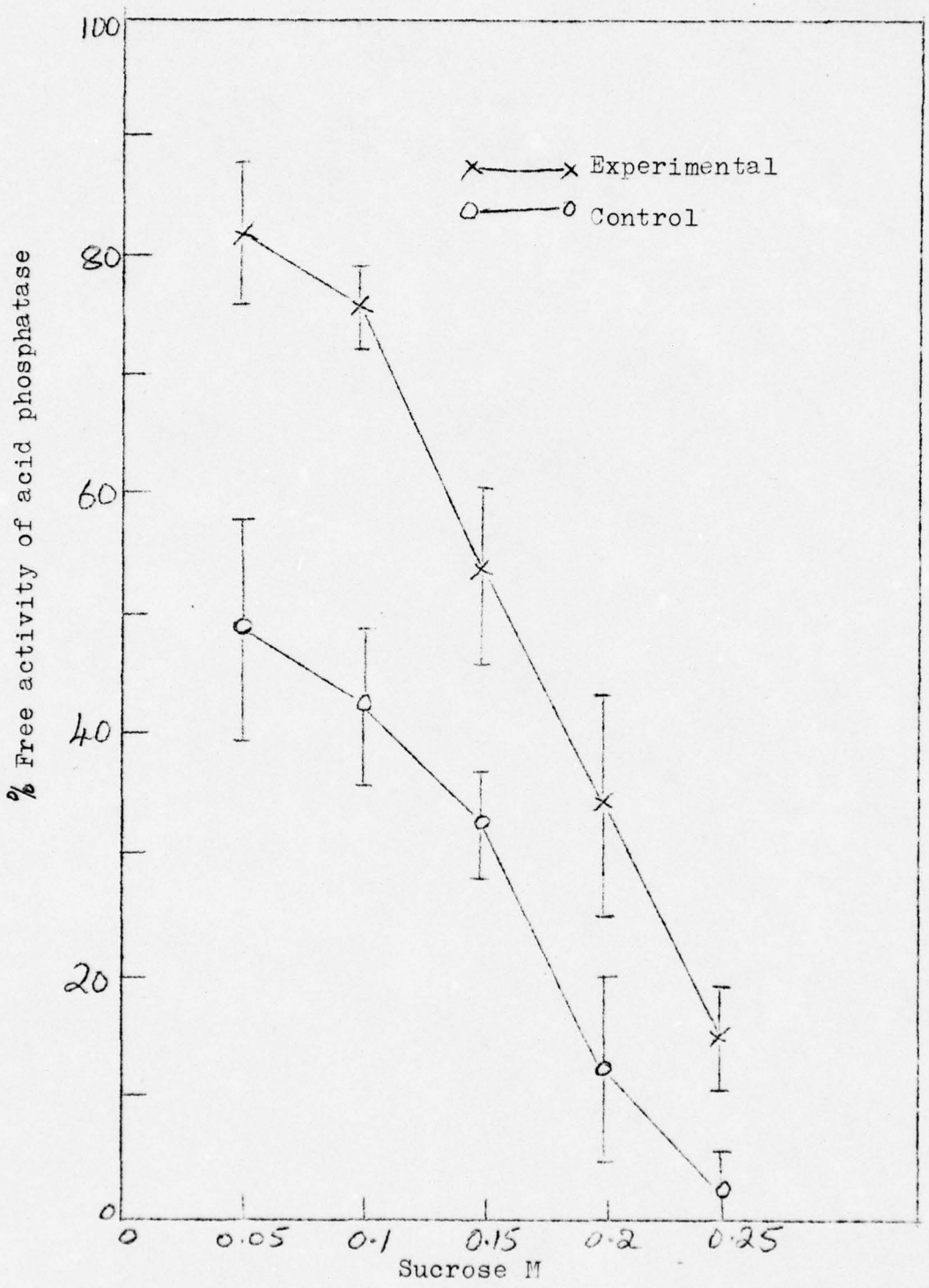


Fig.3: AGGRAVATING INFLUENCE OF OSMOTIC STRESS CONDITIONS ON THE RELEASE OF ACID PHOSPHATASE FROM ISOLATED HEPATIC LYSOSOMAL-RICH FRACTIONS-INFLUENCE OF HYPOBARIA

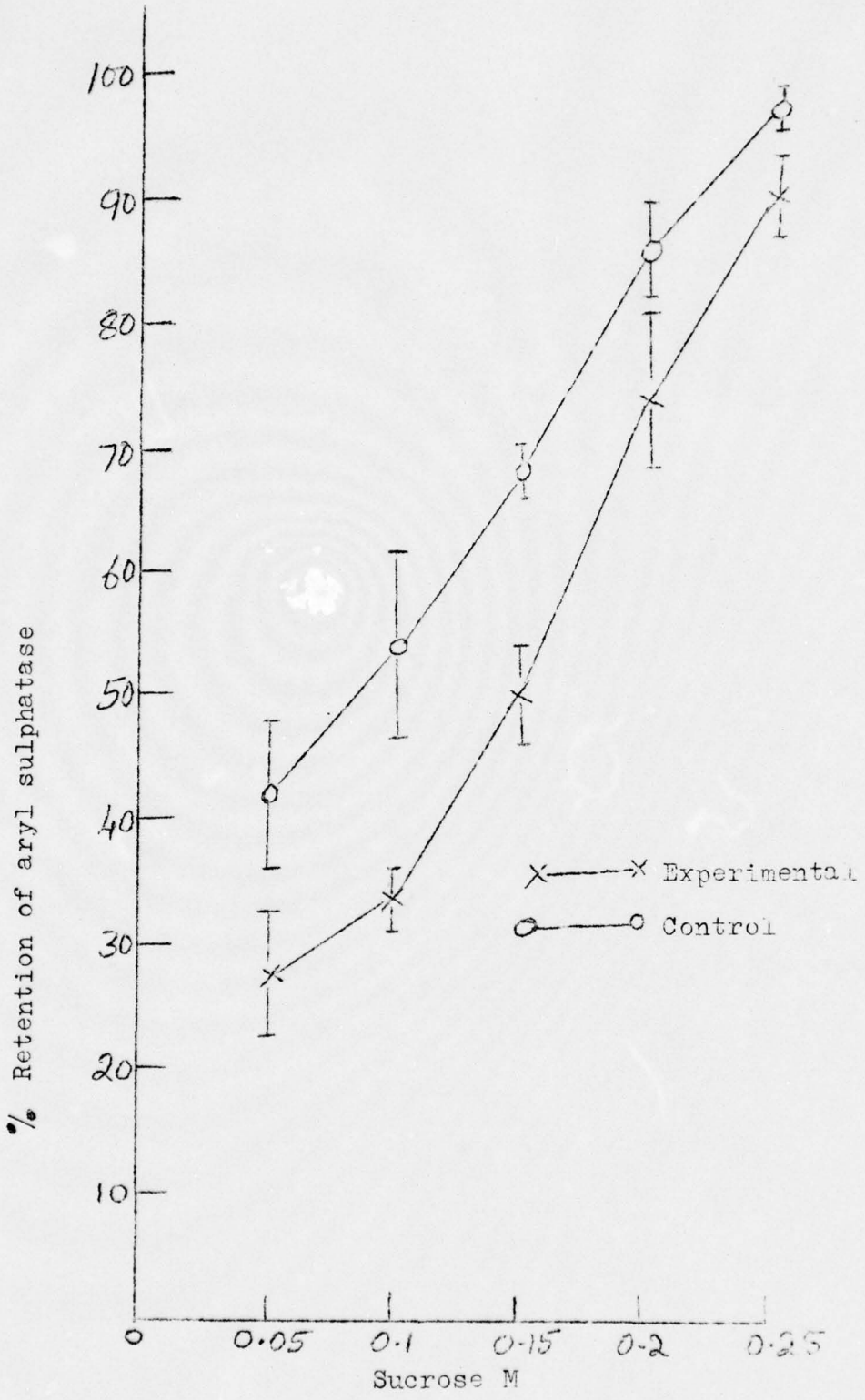


Fig.4: AGGRAVATING INFLUENCE OF OSMOTIC STRESS CONDITIONS ON THE LIBERATION OF ARYL SULPHATASE FROM THE ISOLATED LIVER LYSOSOMAL RICH FRACTIONS-INFLUENCE OF HYPOBARIA

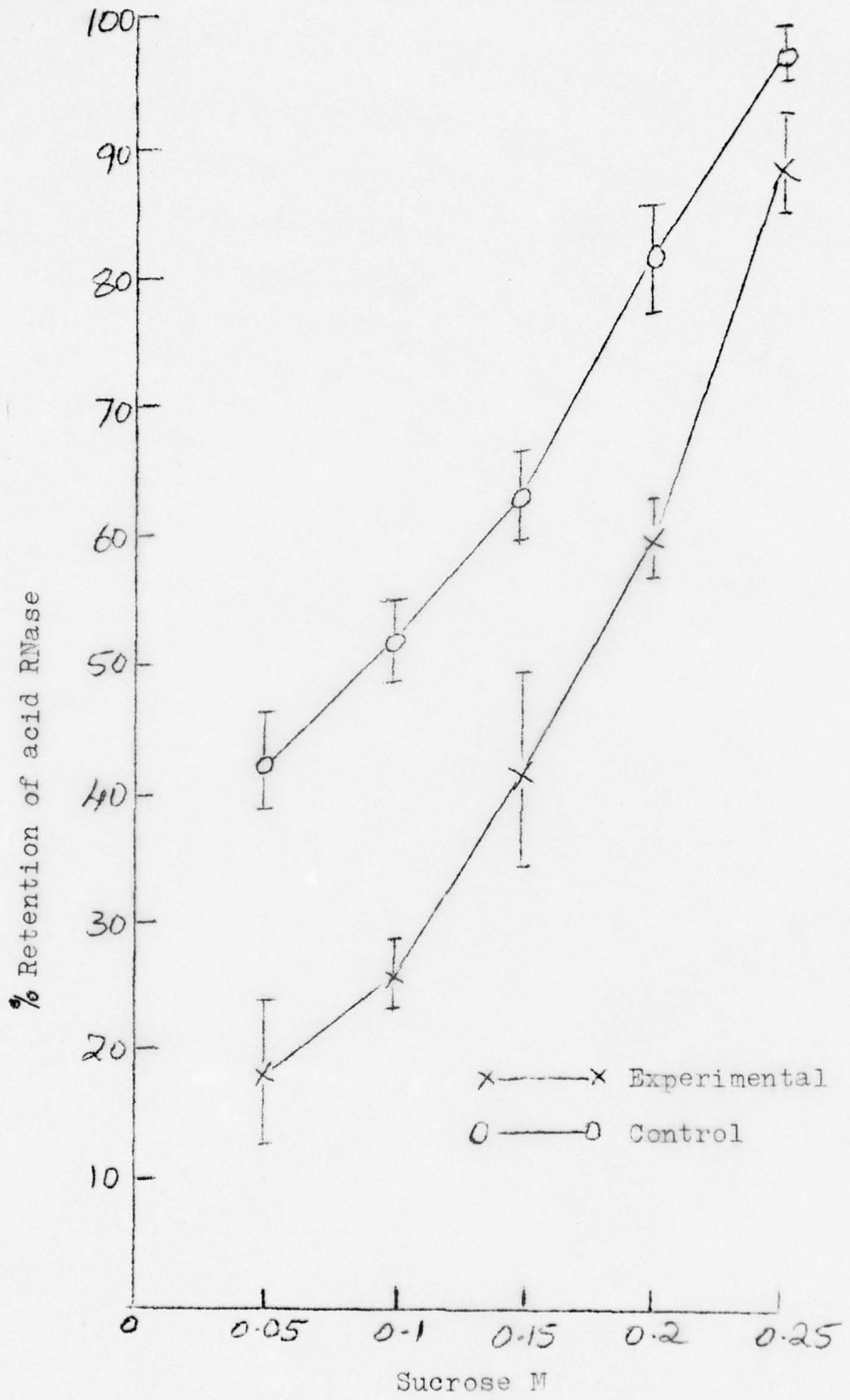


Fig.5: AGGRAVATING INFLUENCE OF OSMOTIC STRESS CONDITIONS ON THE RELEASE OF ACID RNase FROM ISOLATED LIVER LYSOSOMAL-RICH FRACTIONS-INFLUENCE OF HYPOBARIA

Table-6: Effect of high altitude hypoxia on structural latency of acid phosphatase in brain.

Hours of exposure	Total activity$^{\delta}$			Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
	in whole brain	per gm brain					
0	2.95±0.17	1.59±0.13		157±10.3		15.9±2.1	
1	4.21±1.34	2.37±0.65	P < 0.05	185±5.8	P < 0.05	39.4±1.1	P < 0.001
4	5.42±1.02	2.43±0.32	P < 0.05	197±9.4	P < 0.05	41.4±1.8	P < 0.001
12	5.27±0.69	2.29±0.72	P > 0.05	171±9.4	P < 0.05	34.3±1.4	P < 0.001
24	5.07±0.73	2.18±0.51	P < 0.05	204±11.6	P < 0.05	46.6±1.3	P < 0.001
36	4.83±0.53	2.34±0.27	P < 0.05	238±10.4	P < 0.001	32.8±1.7	P < 0.001

δ - Total activity is expressed as µmoles of p-nitrophenol released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of p-nitrophenol released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

P > 0.05 Not significant; P < 0.05 Highly significant.

The values are statistically evaluated by comparing with that of 0 hr exposure as control.

Table-7: Effect of high altitude hypoxia on structural latency of acid ribonuclease in brain.

Hours of exposure	Total activity$^{\delta}$			Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
	in whole brain	Per gm brain					
0	2.34±0.08	1.32±0.069		237±9.4		7.42±1.2	
1	4.13±1.60	2.95±0.673	P < 0.05	297±13.0	P < 0.001	22.90±0.6	P < 0.001
4	3.84±0.04	1.99±0.125	P < 0.001	320±12.3	P < 0.001	37.40±1.3	P < 0.001
12	3.97±0.05	2.07±0.231	P < 0.05	325±15.2	P < 0.001	39.90±2.9	P < 0.001
24	4.09±1.10	2.11±0.139	P < 0.001	377±14.3	P < 0.001	43.70±4.7	P < 0.001
36	3.87±0.06	2.14±0.234	P < 0.001	289±6.3	P < 0.001	32.10±1.1	P < 0.001

δ - Total activity is expressed as µmoles of adenine equivalents released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of adenine equivalents released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

The values are statistically evaluated by comparing with that of 0 hr exposure as control.

P > 0.05 Not significant; P < 0.05 Highly significant.

Table-8: Effect of high altitude hypoxia on structural latency of cathepsin D in brain.

Hours of exposure	Total activity$^{\delta}$			Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
	in whole brain	per gm brain					
0	1.09±0.073	0.605±0.033		99.7±5.8		6.3±0.2	
1	1.24±0.061	0.627±0.024	P 7 0.05	123.0±5.4	P ∠ 0.05	36.4±0.6	P ∠ 0.001
4	1.39±0.043	0.735±0.063	P ∠ 0.05	134.0±5.4	P ∠ 0.01	40.8±2.4	P ∠ 0.001
12	1.37±0.102	0.778±0.039	P ∠ 0.01	145.0±3.1	P ∠ 0.001	47.2±2.4	P ∠ 0.001
24	1.29±0.083	0.792±0.024	P ∠ 0.001	139.0±10.7	P ∠ 0.01	30.7±1.7	P ∠ 0.001
36	1.28±0.045	0.754±0.031	P ∠ 0.001	141.0±6.3	P ∠ 0.001	22.4±3.7	P ∠ 0.01

δ - Total activity is expressed as µmoles of tyrosine equivalents released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of tyrosine equivalents released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

Values are statistically evaluated by comparing with that of 0 hr exposure as control.

P 7 0.05 Not significant; P ∠ 0.05 Highly significant.

Table-9: Effect of high altitude hypoxia on structural latency of aryl sulfatase in brain.

Hours of exposure	Total activity$^{\delta}$			Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
	in whole brain	per gm brain					
0	0.84±0.045	0.47±0.063		110±4.3		4.2±0.8	
1	1.02±0.110	0.56±0.037	P < 0.05	103±3.3	P < 0.05	7.3±0.2	P < 0.01
4	1.17±0.070	0.56±0.051	P < 0.05	131±1.9	P < 0.01	8.8±0.2	P < 0.001
12	1.14±0.050	0.60±0.043	P < 0.05	111±4.5	P > 0.05	9.4±0.5	P < 0.001
24	1.20±0.240	0.62±0.002	P < 0.05	93±2.6	P < 0.01	6.4±1.1	P < 0.05
36	1.07±0.090	0.57±0.037	P < 0.05	113±8.0	P > 0.05	5.7±0.5	P < 0.05

δ - Total activity is expressed as µmoles of p-nitrocatechol released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of p-nitrocatechol released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as Mean ± S.E.M. of 6 observations.

The values are statistically evaluated by comparing with that of 0 hr exposure as control.

P > 0.05 Not significant; P < 0.05 Highly significant.

Autolytic influence of temperature on the lytic susceptibility of liver lysosomal-rich fractions: Accentuation of lytic susceptibility to osmotic stress indicated that exposure to hypobaric hypoxia seemed to modify in such a way as to liberate more soluble enzymes from lysosomal matrix. As explained in Materials and Methods the lysosomal-rich fractions isolated from rats exposed to simulated high altitude hypoxia for 4 hr. were incubated at 37°C for different time intervals to evaluate the influence of time-course autolysis. Figs.VI and VII show that the time-course autolysis accelerated the liberation of acid phosphatase respectively into supernatant.

Influence of ionic strength on the solubility of acid phosphatase: Influences of osmotic stress and time-course autolysis on the liberation of lysosomal enzymes from isolated lysosomal-rich fractions from liver of experimental animals under hypobaric hypoxia indicated the probable modification of lysosomes in experimental animals. Hence, it was of interest to investigate the effect of change in ionic strength on the liberation of acid phosphatase into soluble particulate-free supernatant from liver lysosomal-rich fractions of experimental animals. Fig.VIII depicts the influence of increasing ionic strength on the solubility of acid phosphatase from liver lysosomal-rich fractions.

Permeability of lysosomes to substrates: In the light of the above studies, it was desired to find out whether permeability property of lysosomes in experimental animals has been modified. Table-10 indicated that no alteration in permeability property had occurred as evidenced by the failure of p-nitrophenyl phosphate to elicit the release of lysosomal enzymes. Therefore, it appears as if there is no modification in permeability characteristics.

Solubility of lysosomal enzymes: Various disruptive forces incite the liberation of enzymes from lysosomal matrix. Studies were carried out to evaluate the efficiency of accentuation of solubility of enzymes by various disruptive

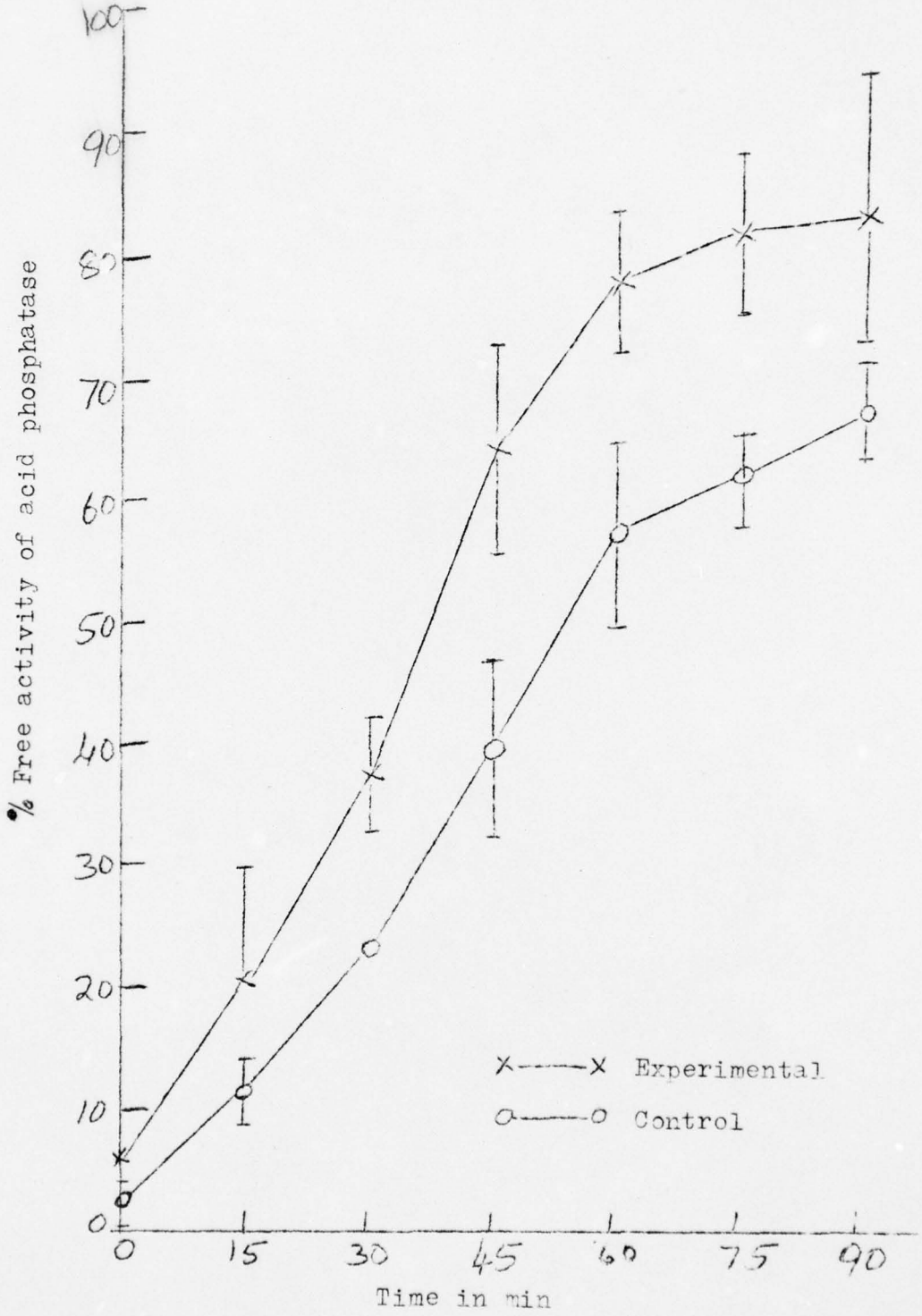


Fig.6: AGGRAVATING INFLUENCE OF AUTOLYSIS ON THE LIBERATION OF ACID PHOSPHATASE FROM THE ISOLATED LYSOSOMAL-RICH FRACTIONS-INFLUENCE OF HYPOBARIA

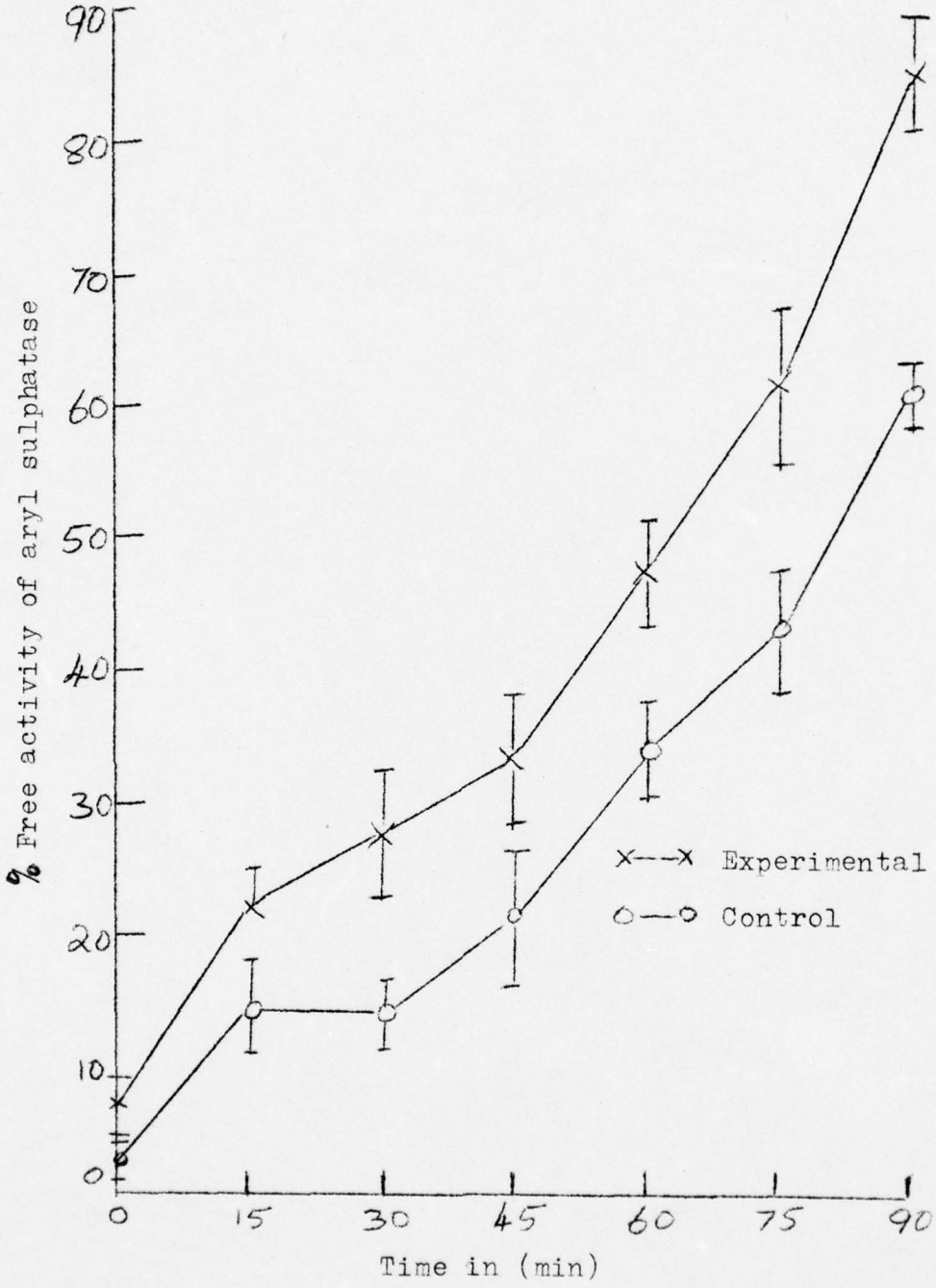


Fig.7: AGGRAVATING INFLUENCE OF AUTOLYSIS ON THE LIBERATION OF ARYL SULPHATASE FROM ISOLATED HEPATIC LYSOSOMAL-RICH FRACTIONS - INFLUENCE OF HYPOBARIA.

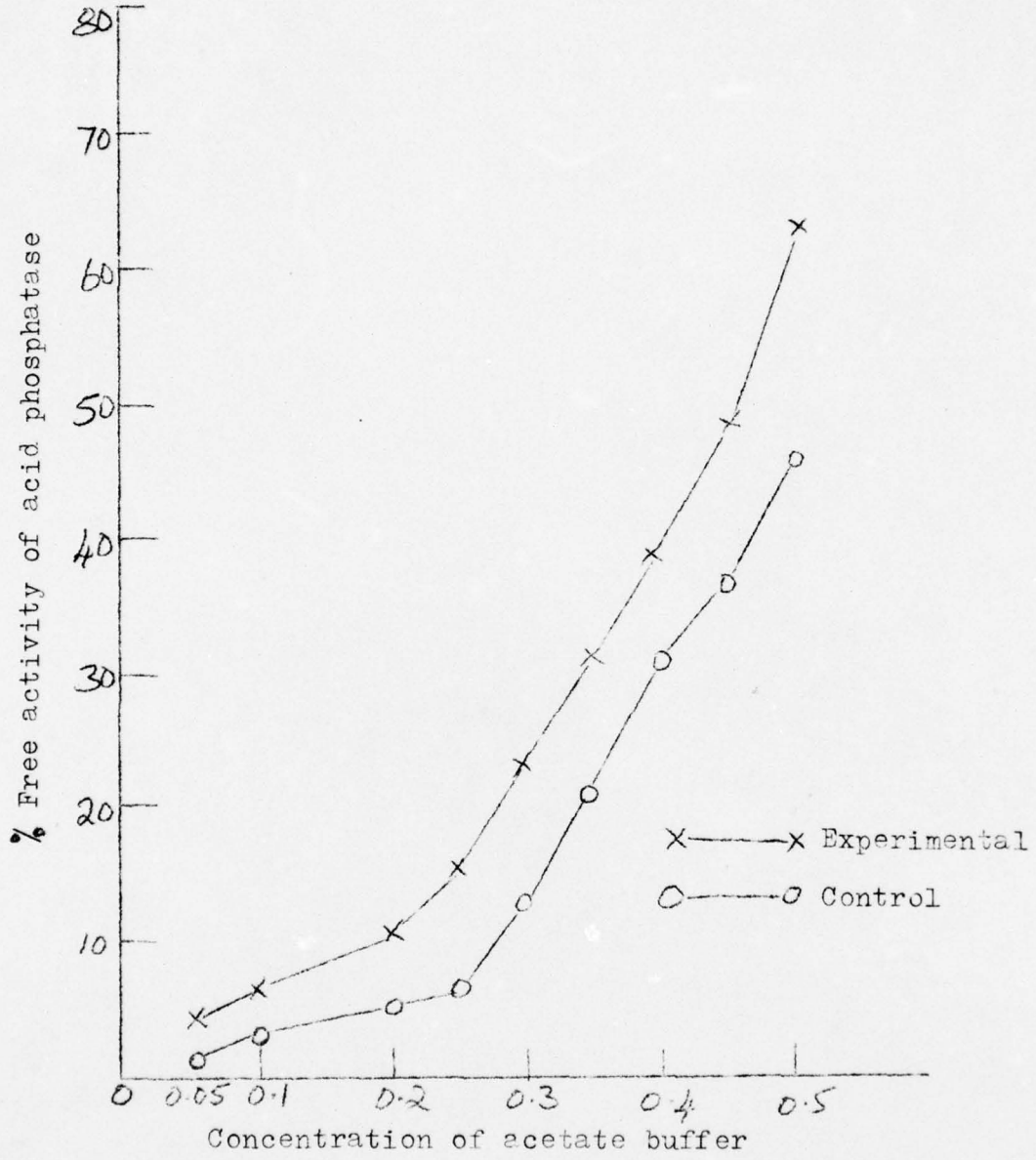


Fig.8: EFFECT OF IONIC STRENGTH ON THE SOLUBILITY OF ACID PHOSPHATASE FROM THE ISOLATED LIVER LYSOSOMAL-RICH FRACTIONS-INFLUENCE OF HYPOBARIA

agents such as Triton X-100, freezing and thawing and exposure to hypotonic sucrose solution (Table-11) reveals that Triton X-100 at a concentration of 1 mg/0.1 ml release submaximal amount of acid phosphatase. Triton X-100 elicited the maximal disruption amongst these agents.

DISCUSSION

A response is a direct reaction, either adaptive or non-adaptive to an environmental stimulus. Organisms subjected to stress conditions adapt themselves to increase their survival efficiency in the new environment. Hence, the organisms should evoke the potential regulatory mechanisms for their survival efficiency. This involves a series of coordinated changes at physiological, behavioural and biochemical levels. The adaptation strategy an organism adopts depends on temporal factor and intensity of stressor.

Biochemical alteration is effected by appropriate modulation of various metabolism under the influence of stressor. These modulations may be elicited through the mediation of hormones or other homeostatic regulators, which play paramount role in the maintenance of internal milieu constant. Pervasive effects of high altitude conditions which include oxygen impoverished environment would call for the changes at the biochemical levels. Simulated high altitude with simultaneous influences of hypobaria, hypoxia and hypothermia cause many biochemical changes. The prime factor in alleviating the stress conditions are the catabolism of unwanted cellular materials, enhanced synthesis of RNA and proteins and induction of wide variety of regulatory enzymes, mainly catabolic enzymes like tyrosine amino transferase, tryptophan pyrrolase etc. Considering the magnitude of modifications occurring under hypobaric hypoxia it is easily conceivable that poised system such as lysosomal system may participate actively in the maintenance of homeostasis. Taking into account the fact that extensive autophagy occurred within 30 min of anoxia in rat /brain (Ericsson, 1969) and enhanced fragility of lysosomes of liver and

brain (Tables 1 to 9) surmised that activation of lysosomes may be an expression of physiological survival mechanism. At this stage it is very premature to conclude that such an influence of **hypobaric** hypoxia on lysosomes is the primary event. It may very well be that enhanced fragility consequent to increased autophagy may be a secondary event.

Fig.I and Tables 1 to 9 clearly implied the loss of structural latency. Such loss in structural latency could stem from various reasons. The increase in percent free activity may arise from the enhanced fragility of lysosomes to homogenization procedure. Nevertheless, increase **in** / synthesis of these catabolic enzymes. The consequence of the above implication is understandable since lysosomal system is known to take active part in the maintenance of homeostasis. /total activity indicates that there may be an increase in

Diminished structural latency of lysosomal enzymes induced by high altitude hypoxia stress may stem from various possibilities: (i) increased enzyme synthesis, (ii) inefficient cellular disruption during homogenization process combined with preferential lysis of tissues of experimental animals, (iii) disruption of lysosomes or increased fragility of these organelles and (iv) increased permeability of the lysosomal membrane to enzyme substrates. Possibility of **increased** permeability of the **membrane** to enzyme substrates is not tenable in this case because of the fact that the methodology employed in determining the free activity involves the assay of activities in the non-sedimentable particulate-free supernatant and not at the homogenate level. Moreover, the data depicted in Table-11 clearly rules out this possibility of increased permeability leading to more free activity than in control. At present, equivocalness of the experimental approach does not permit any decisive conclusions to be drawn, regarding the causative factor per se for this collateral enhancement of free activities of enzymes. It is impossible to distinguish whether the liberation of enzymes it caused in vivo under the influence of high altitude

hypoxia or *in vitro* due to mechanical stress of homogenization technique aggravating the release of enzymes from erstwhile sequestered in the lysosomal membrane in experimental animals. In the prevailing situation, the presumptive evidences seem to indicate that the mechanical stress due to homogenization leads to covert labilization of lysosomal enzymes. However, present day biochemical tools are not too penetrating to rule out the possible labilization *in vivo* prior to homogenization. Lysosomal enzymes need not be extruded from the membrane for the degradation. Such a degradation can be achieved by fusing with autophagic vacuoles. The digestion of materials can be carried out in the autophage-lysosomes. Fragility of lysosomes obtained from experimental animals to mechanical stress induced by homogenization technique may be due to enlarged lysosomes.

Such an increase in size should be reflected by more proneness to lysis on exposure to hypotonic solution than the lysosomes obtained from control animals. To verify this hypothesis liver lysosomal-rich fractions isolated from experimental and control rats were exposed to hypotonic sucrose solution. It is evident from Figs.III to V that osmotic stress, indeed, aggravates the lytic susceptibility. Hence, it appears that enlargement in size of liver lysosomal-rich fractions may be responsible for the readiness to liberate the lysosomal enzymes in the case of experimental animals. The augmented release of enzymes into supernatant from the bound form may be conceived as arising either out of damaged lysosomes during homogenization or enhanced substrate permeability. The latter hypothesis advocates the liberation of enzymes from intact lysosomes. Enhanced substrate permeability is ruled out in the present investigation from the results recorded in Table-11. It is further supported by experimental evidences obtained regarding the permeability of rat liver lysosomes to β-glycerophosphate (deDuve, 1965), rat liver α-glucosidase to mannose (Lloyd, 1969) and rat liver preparations to various substrates (Baccino and

Zuretti, 1975; Baccino et al.,1971). Baccino et al.(1971) and Baccino and Zuretti (1971) from their studies proposed that the enzyme from lysosomes either remain attached to sedimentable portions of lysosomes or adsorb secondarily adsorb on other particulate material. Herein one interesting point which attracts attention is that the rate of fall in percent retention for acid phosphatase appears to be the same except for the initial 'drop' in experimental animals. This decrease in percent retention of acid phosphatase in experimental animals may be due adsorption artifact. However, the complexity of situation precludes any firm conclusion to be derived except that the augmentation of lytic susceptibility to osmotic stress lends further support to the enhanced fragility of lysosomes prepared from liver of experimental rats.

Further corroborative evidences for the percent findings that hypobaric hypoxia alters the structural latency of lysosomal enzymes were provided by exacerbating stimulus of thermal autolysis on isolated lysosomal-rich fractions. Figs.VI and VII depict the influence of incubation at 37°c for different time intervals on the augmented liberation of acid phosphatase from lysosomes of experimental animals. The striking parallelism exhibited by aryl sulfatase and acid phosphatase can be conjectured that a common mechanism underlies the accentuated liberation of all these enzymes. During progressive autolysis due to incubation at 37°C as also due to exposure to hypotonic sucrose solution a close correlation of release of these enzymes at an enhanced rate to the particulate-free supernatant can be observed. It may be attributed to high altitude hypoxic conditions. The experimental design involved rapid chilling and removal of particulate from supernatant, the estimation of enzyme activities being carried out both in particulate-free supernatant and particulate fractions.

Another interesting point which deserves attention is that of these stimuli liberating enzymes from lysosomes,

Triton X-100 elicits maximal accentuating influence on the release of enzymes from lysosomes. Hence, the percent free activity of the enzymes and the percent retention were computed on the basis that Triton X-100 (1 mg/0.1 ml) released enzyme activities as 100 percent. It is significant to observe that Triton X-100 (1 mg/0.1 ml) which was included in the incubation mixture to determine the total activity from the particulate fraction, even for 1 hr. left behind some residual protein if the reaction mixture was centrifuged.

Documented evidences exist to implicate lysosomes in playing preponderate role in cellular economy (deDuve and Wattiaux, 1966). Hence, with the present evidence it may be surmised that increase in percent free activities connotes the involvement of lysosomes as an expression of physiological survival mechanism.

In order to appreciate the physiological significance of the increase in catabolic activity, one should know the functional role of the lysosomal system as to whether it is merely to scavange the unwanted portions of cellular material or to actively participate in various metabolisms. Acid DNase and acid RNase have been implicated to hydrolyze DNA and RNA, respectively as a result of accumulation of carcinogenic agents within lysosomes consequence of which leads to rupture of lysosomes (Allison, 1969). It appears that lysosomes participate in the metabolism of various tissue components (Dean, 1975). One could predict from the above observations that lysosomal system can serve both to scavange the unwanted portions of cellular material and to participate in metabolism. It is of relevance to point out that cathepsin D, acid RNase and acid phosphatase of liver and brain, and β-glucuronidase in liver are drastically affected. Another important question whether the effect of hypobaric hypoxia induced alteration in structural integrity is quite specific to lysosomal membrane. Under hypobaric hypoxic conditions in low land human beings to high altitude results in the change in many parameters such as erythrocyte count, erythrocyte

membrane, mitochondria etc.(Frisancho, 1975). However, the interactions between organisms and their environment are exceedingly complex and no single limiting mechanism exists for one environmental parameter in a given species.

In conclusion, it appears that lysosomal system is triggered into action as a secondary event.

Role of lysosomes during liver regeneration

Introduction

Repair by regeneration is a biological phenomenon experienced in varying degree by all members of the phylogenetic series. It signifies the replacement or restoration of lost parts, including cells, by structurally, and often functionally similar units. The adult mammalian liver has a remarkable capacity for growth by compensatory hypertrophy and hyperplasia after the loss of functional hepatic tissue. It is usually a stable relatively quiescent tissue, yet growth exceeding that of embryonic or malignant cells can be initiated by a variety of experimental procedures. When part of the liver is removed or damaged, many changes take place in the remnant, some of which are relevant to the proliferative response and some of which occur as a result of an expression of the profound metabolic and other changes which accompany this major event. Thus, there is apparently a large reserve of functional capacity of the liver because experimental animals can survive the removal of upto 80 percent of the liver without untoward effect.

During the initial 8 hr. following the partial hepatectomy there was a dispersion of cytoplasmic basophilia within hepatic cell cytoplasm, particularly in the periphery of hepatic lobules (Fischer, 1969). Intracytoplasmic droplets also occurred during this early period. These observations appeared to be related to dilatations of granular endoplasmic reticulum as well as apparent relocation of granules from endoplasmic reticulum to cytoplasm. The disposition of the granular ER during early phases of regeneration was, in large

part, dependent upon the glycogen content of the hepatic cells. During early period glycogen depletion and lipid accumulation were encountered. Hence, during glycogen depletion the granular ER were poorly oriented. An increase in size of hepatic cells, nuclei and nucleoli was observed between 12 and 24 hr. following partial hepatectomy. Mitosis took place at 24 hr. Their number increased rapidly upto 28 hr. followed by a rapid decline and subsequently more gradual decrease during the next several days.

Cytoplasmic inclusion, most often comprised of solitary limiting membranes and a variable matrix corresponding to microbodies and cytosomes are increased during the first 18 hr. following partial hepatectomy [Fischer (1969); Bresnick, 1971]. The lysosomal nature of many of these structures is confirmed by their lysosomal content (Essner and Novikoff, 1961). An increase in lysosomal content has been established by Adams (1963). Increase in autophagy has been recorded during regeneration indicating the probable involvement of lysosomal system in the preparation for cell division (Bresnick, 1971). Lysosomes have been localized around nucleus and mitotic spindle during cytokinesis (Bresnick, 1971; Lane and Becker, 1967). This implies the active participation of lysosomes in mitosis occurring in liver regeneration. It is supported by the fact that lysosomes are activated during nuclear activity.

Generally, no abnormality is seen during regenerative process. At 72 hr. following partial hepatectomy, hepatic cells appear normally ultrastructurally.

Synthesis of RNA and DNA and protein increased during rapid cell multiplication in regenerating rat liver (Short *et al.*, 1972; Ord and Stocken, 1972; Ferris and Clark, 1972). Chatterjee *et al.*(1974) have shown that lysosomal acid phosphatase and β-glucuronidase and alkaline phosphatase increased during liver regeneration. Further, cAMP and ACTH enhanced the total activites of these enzymes in regenerating rat liver.

It is of interest to study the role of lysosomes under regenerating conditions such as hepatic regeneration after partial hepatectomy. Regeneration is affected by environmental parameters. Here, lysosomes of regenerating rat liver were studied in animals, which were exposed to high altitude hypoxia for 24 hr. prior to partial hepatectomy.

Materials and Methods

Substrates and other chemicals are as described earlier.

Experimental set up: Male Wistar rats of Institute colony were fed with rat pellet diet (Hindustan Lever Ltd., Bombay). They were given water ad libitum. Rats weighing 120 g were divided into 8 groups. Groups I to III were normobaric and kept at room temperature. Groups II and III were subjected to partial hepatectomy according to Higgins and Anderson (1931) and allowed to regenerate for 24 and 48 hr. respectively. Groups IV and VIII were exposed to a simulated high altitude as described earlier for 24 hr. Groups VII and VIII were then subjected to partial hepatectomy and allowed to regenerate for 24 and 48 hr. respectively. Animals of Group IV were sacrificed immediately after exposure to high altitude hypoxia. Rats of Groups V and VI were brought back to room temperature and pressure, after exposure to high altitude hypoxia for 24 and 48 hr. respectively.

Partial hepatectomy was performed as follows: Large median and left lateral lobes of the liver were ligated and excised leaving the right lateral and caudal lobes intact. Total excised liver usually amounted to about 70 percent. After surgical removal of part of the liver the incision is sutured and clipped.

Animals were killed by decapitation and livers were excised and the portions of liver which underwent necrosis were removed.

Preparation of tissue fractions, enzyme assays and the mode of calculation were as detailed earlier.

RESULTS

Table-1 presents the liver weights during regeneration for groups II, III, VII and VIII. The rate of weight gain in rats exposed to hypobaric hypoxia prior to partial hepatectomy (Groups VII and VIII) was less than that in the normobaric rats (Groups II and III). This may indicate that hypobaric hypoxia affects regeneration.

Tables-2 to 6 show the total activities in whole and gram liver and total specific and percent free activities of acid phosphatase, aryl sulfatase, β-glucuronidase, cathepsin D and acid RNase in regenerating rat. From the Tables it is evident that there is increase in percent free activity of these enzymes in rat livers where regeneration occurs. Data shown in Tables divulge that structural latency of lysosomes of regenerating rat liver was lost regardless of whether rats were exposed to hypobaric hypoxia for 24 hr prior to partial hepatectomy or normobaric rats.

Percent free activities of lysosomal enzymes in Groups V and VI are elevated as compared to Group I. However, these were less as compared to Group IV.

Tables 2 to 6 reveal the increase in total activities per g liver of these enzymes for groups II to VIII over group I. However, increments in total activities in Groups VII and VIII were less than in groups II and III, respectively. Another observation which deserves attention is that total activities per gram liver was more than that of groups VII and VIII. If the increment in total activities of these enzymes/g liver was considered to reflect the increase in synthesis then there occurred enhanced synthesis of these enzymes in regenerating liver obtained from normobaric rats(Groups II and III). Further, exposure to high altitude hypoxia affected the regeneration rate.

DISCUSSION

Physiological regeneration encompasses the phenomenon of cellular replacement encountered in growth and aging. It is

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Table-1: Effect of high altitude hypoxia on liver regeneration.

Hours after hepatectomy	Normobaric rate (wt.of liver in g)	Rate of growth (percent)	Hypobaric rate(wt.of liver in g)	Rate of growth (percent)
0	1.57 ± 0.07$^{\delta}$		1.64 ± 0.14	
24	2.137± 0.021	36.0	1.763± 0.034	7.5
48	2.417± 0.047	12.9	1.997± 0.076	12.0

Normobaric rats were hepatectomized and allowed for regeneration for 24 and 48 hours respectively. Hypobaric rats were exposed to high altitude hypoxia for 24 hours prior to hepatectomy. After hepatectomy they were allowed to regenerate for 24 and 48 hours. Liver weights were determined in both the groups.

Table-2: Effect of liver regeneration on acid phosphatase

Group	Total activity$^{\delta}$ per gm liver		Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
I	6.32±1.23		329±17.8		12.0±1.7	
II	8.52±0.72	P < 0.05	427±15.4	P < 0.01	25.4±1.9	P < 0.001
III	8.94±1.49	P > 0.05	435±20.9	P < 0.01	17.2±2.4	P < 0.05
IV	8.76±0.91	P < 0.001	410±16.7	P < 0.01	39.8±3.2	P < 0.001
V	7.89±1.04	P > 0.05	398±12.5	P < 0.05	29.4±3.6	P < 0.01
VI	8.24±0.79	P < 0.05	354±16.3	P > 0.05	28.6±2.8	P < 0.001
VII	7.43±1.73	P > 0.05	379±14.3	P < 0.05	36.7±1.7	P < 0.001
VIII	7.62±1.09	P > 0.05	384±16.4	P < 0.05	28.7±4.6	P < 0.01

δ - Total activity is expressed as µmoles of p-nitrophenol released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of p-nitrophenol released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 6 observations.

Values are statistically evaluated by comparing with that of Group I.

Groups I-III are normobaric; II and III - hepatectomized and allowed for regeneration for 24 and 48 hr. respectively.

Groups IV-VIII were exposed to high altitude hypoxia for 24 hr.; VII and VIII - hepatectomized and allowed for regeneration for 24 and 48 hr. respectively; V and VI - brought back to room temperature and pressure for 24 and 48 hr. respectively.

P > 0.05 Not significant; P < 0.05 Highly significant.

Table-3: Effect of liver regeneration on acid ribonuclease.

Group	Total activity$^{\delta}$ per gm liver		Total specific activity$^{\delta\delta}$		Percent free activity$^{\delta\delta\delta}$	
I	2.07±0.16		459±7.4		7.6±1.8	
II	4.92±0.37	P < 0.001	594±9.7	P < 0.001	21.7±0.9	P < 0.001
III	5.72±0.12	P < 0.001	647±10.6	P < 0.001	34.5±6.5	P < 0.001
IV	5.20±0.29	P < 0.001	604±15.2	P < 0.001	37.6±9.0	P < 0.01
V	4.49±0.34	P < 0.001	585±13.0	P < 0.001	28.5±8.5	P < 0.05
VI	4.25±0.65	P < 0.001	527±14.2	P < 0.01	30.1±6.2	P < 0.01
VII	3.90±0.14	P < 0.001	487± 9.6	P < 0.05	29.4±4.6	P < 0.001
VIII	4.30±0.24	P < 0.001	495±10.4	P < 0.05	27.6±3.9	P < 0.001

δ - Total activity is expressed as µmoles of adenine equivalents released per min.

$\delta\delta$ - Total specific activity is expressed as nmoles of equivalents released per mg protein per incubation time.

$\delta\delta\delta$ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 4 observations.

The values are statistically evaluated by comparing with that of Group I.

Groups I-III - Normobaric; II and III - Hepatectomized and allowed for regeneration for 24 and 48 hr respectively.

Groups IV-VIII - Exposed to high altitude hypoxia for 24 hr.; VII and VIII - Hepatectomized and allowed for regeneration for 24 and 48 hr. respectively; V and VI - Brought back to room temperature and pressure for 24 and 48 hr. respectively.

P > 0.05 Not significant; P < 0.05 Highly significant.

Table-4: Effect of liver regeneration on cathepsin D.

Group	Total activity[δ] per gm liver		Total specific activity[δδ]		Percent free activity[δδδ]	
I	0.93±0.12		189±10.7		3.7±1.9	
II	1.47±0.26	P < 0.05	243±11.6	P < 0.01	27.4±2.6	P < 0.001
III	1.65±0.34	P < 0.05	277± 9.6	P < 0.001	29.5±1.8	P < 0.001
IV	1.45±0.11	P < 0.01	283±13.1	P < 0.001	32.4±2.7	P < 0.001
V	1.25±0.07	P < 0.05	249±11.9	P < 0.01	23.7±1.7	P < 0.001
VI	1.32±0.10	P < 0.05	256± 5.8	P < 0.001	22.4±0.6	P < 0.001
VII	1.07±0.13	P > 0.05	221± 8.4	P < 0.05	29.4±0.4	P < 0.001
VIII	1.20±0.19	P > 0.05	272±11.6	P < 0.001	23.8±0.5	P < 0.001

δ - Total activity is expressed as μmoles of tyrosine equivalents released per min.

δδ - Total specific activity is expressed as nmoles of tyrosine equivalents released per mg protein per incubation time.

δδδ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms) x 100.

Values are expressed as mean ± S.E.M. of 4 observations.

The values are statistically evaluated by comparing with that of Group I.

Groups I-III - Normobaric; II and III - Hepatectomized and allowed for regeneration for 24 and 48 hr. respectively.

Groups IV-VIII - Exposed to high altitude hypoxia for 24 hr.; VII and VIII - Hepatectomized and allowed for regeneration for 24 and 48 hr. respectively; V and VI - Brought back to room temperature and pressure for 24 and 48 hr. respectively.

P > 0.05 Not significant; P < 0.05 Highly significant.

Table-5: Effect of liver regeneration on aryl sulfatase.

Group	Total activity[δ] per gm liver		Total specific activity[δδ]		Percent free activity[δδδ]	
I	3.8±0.41		275±14.7		4.0±0.2	
II	5.1±0.39	P < 0.01	325±11.9	P < 0.05	10.7±0.8	P < 0.001
III	6.0±0.17	P < 0.001	379±14.9	P < 0.001	8.4±0.6	P < 0.001
IV	4.7±0.40	P < 0.05	394±11.4	P < 0.001	10.4±0.4	P < 0.001
V	4.0±0.39	P > 0.05	345±14.4	P < 0.01	8.7±1.2	P < 0.01
VI	4.3±0.13	P > 0.05	364±12.3	P < 0.001	8.5±0.3	P < 0.001
VII	3.9±0.27	P > 0.05	375± 9.4	P < 0.001	12.4±0.17	P < 0.001
VIII	4.2±0.03	P > 0.05	325± 8.7	P < 0.05	10.9±0.1	P < 0.001

δ - Total activity is expressed as μmoles of p-nitrocatechol released per min.

δδ - Total specific activity is expressed as nmoles of p-nitrocatechol released per mg protein per incubation time.

δδδ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms)x100.

Values are expressed as mean ± S.E.M. of 4 observations.

The values are statistically evaluated by comparing with that of Group I.

Groups I-III - Normobaric; II and III - Hepatectomized and allowed for regeneration for 24 and 48 hr.respectively.

Groups IV-VIII - Exposed to high altitude hypoxia for 24 hr; VII and VIII - Hepatectomized and allowed for regeneration for 24 and 48 hr. respectively; V and VI - Brought back to room temperature and pressure for 24 and 48 hr. respectively.

P > 0.05 Not significant; P < 0.05 Highly significant.

Table-6: Effect of liver regeneration on β-glucuronidase.

Group	Total activity[δ] per gm liver		Total specific activity[δδ]		Percent free activity[δδδ]	
I	2.94±0.23		87± 7.1		4.9±1.2	
II	5.98±0.12	P < 0.001	128±10.5	P < 0.01	22.4±3.7	P < 0.001
III	6.07±0.53	P < 0.001	343±10.7	P < 0.001	27.3±4.1	P < 0.001
IV	4.67±0.63	P < 0.05	270± 6.23	P < 0.001	12.4±1.8	P < 0.01
V	4.38±0.72	P < 0.01	240± 6.43	P < 0.001	8.4±0.8	P < 0.05
VI	4.39±0.36	P < 0.05	254± 6.60	P < 0.001	8.0±0.7	P < 0.05
VII	3.21±0.31	P > 0.05	217± 0.37	P < 0.001	14.9±0.5	P < 0.001
VIII	3.42±0.40	P > 0.05	207± 0.31	P < 0.001	23.9±1.2	P < 0.001

δ - Total activity is expressed as μmoles of phenolphthalein released per min.

δδ - Total specific activity is expressed as nmoles of phenolphthalein released per mg protein per incubation time.

δδδ - Percent free activity is calculated as (Total activity in free form/Total activity in bound and free forms)x100.

Values are expressed as mean ± S.E.M. of 4 observations.

The values are statistically evaluated by comparing with that of Group I.

Groups I-III - Normobaric; II and III - Hepatectomized and allowed for regeneration for 24 and 48 hr. respectively.

Groups IV-VIII - Exposed to high altitude hypoxia for 24 hr; VII and VIII - Hepatectomized and allowed for regeneration for 24 and 48 hr. respectively; V and VI - Brought back to room temperature and pressure for 24 and 48 hr. respectively.

P > 0.05 Not significant; P < 0.05 Highly significant.

axiomatic that the ability of tissues to exhibit such physiological regeneration is inversely proportional to their degree of differentiation. Organs such as liver, kidney and connective tissue in general, show a low mitotic index (Fischer, 1969). Such cells, however, do exhibit the capacity for accelerated renewal following partial removal.

The application of such techniques as labelling with ^{3}H-thymidine and mitotic arrest by colchicine has provided accurate estimates of the renewal velocity of various cell populations. Edwards and Klein (1961) extensively studied the relative renewal rates of 34 different types of cells in normal adult mouse with ^{3}H-thymidine. They concluded that rigid categorization of cell types according to mitotic index in unwarranted, since all cells, save neurons and cardiac muscle, exhibit DNA synthesis at a fairly steady state above that required for normal growth.

Various theories on regenerative stimulus have been propounded, yet, factor(s) stimulating regeneration remains controversial. Nevertheless, whatever be the stimulus for regeneration tissue destruction appears to be the primary event. It has been proposed that the initial catabolism paves the way for unopposed growth activity mediated by other stimuli for instance oestrogens (Szego, 1974), lysosomes may be invoked to play a role in massive degradation of cellular constituents. Here, it is relevant to note that in the present investigation, enhanced free activities of acid phosphatase, acid ribonuclease, β-glucuronidase and cathepsin D were observed in regenerating rat livers both in exposed to simulated high altitude conditions and in normobaric rats (Tables 2 to 6). It has been shown that within 18 hr. DNA synthesis reaches maximum and mitosis commences at 14 hr. (Fischer, 1969; Bresnick, 1971). During mitosis it has been documented that lysosomes play a preponderate role (Szego, 1974? Allison, 1969). These observations can be reconciled by the present finding that percent free and total activities of these enzymes increased.

If the extent of regeneration is measured on the rate of increase in weight, then Table-1 reveals that the exposure to high altitude hypoxia prior to hepatectomy has diminished the regeneration rate.

It is well known that during regeneration, cell population increases and, hence, increase in lysosomal content can be accounted for in normobaric-hepatectomized rats. Chatterjee et al.(1974) have shown that total activities of acid phosphatase, β-glucuronidase and alkaline phosphatase have increased in regenerating rat liver.

Decrease in total activities of lysosomal enzymes in Groups VII and VIII as compared to Groups II to VI can be reconciled if one considers that the rate of regeneration is affected by exposure to hypobaric hypoxia.

Physiological significance of increased fragility of lysosomes as indicated by the loss of structural latency of lysosomal enzymes appears to implicate the involvement of lysosomes in catabolism of tissue components prior to synthetic activity. Ultrastructural data show the autophagy in the intial stages. It is appropriate to point out that percent free activities of cathepsin D, acid RNase, acid phosphatase and β-glucuronidase are at high level indicating the extensive loss of structural latency in regenerating rat liver. Lysosomes may discharge the function of active catabolism of the components so that the constituents can further be used for synthesis of essential components. However, lysosomal activation may not be the primary event as a result of partial hepatectomy.

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