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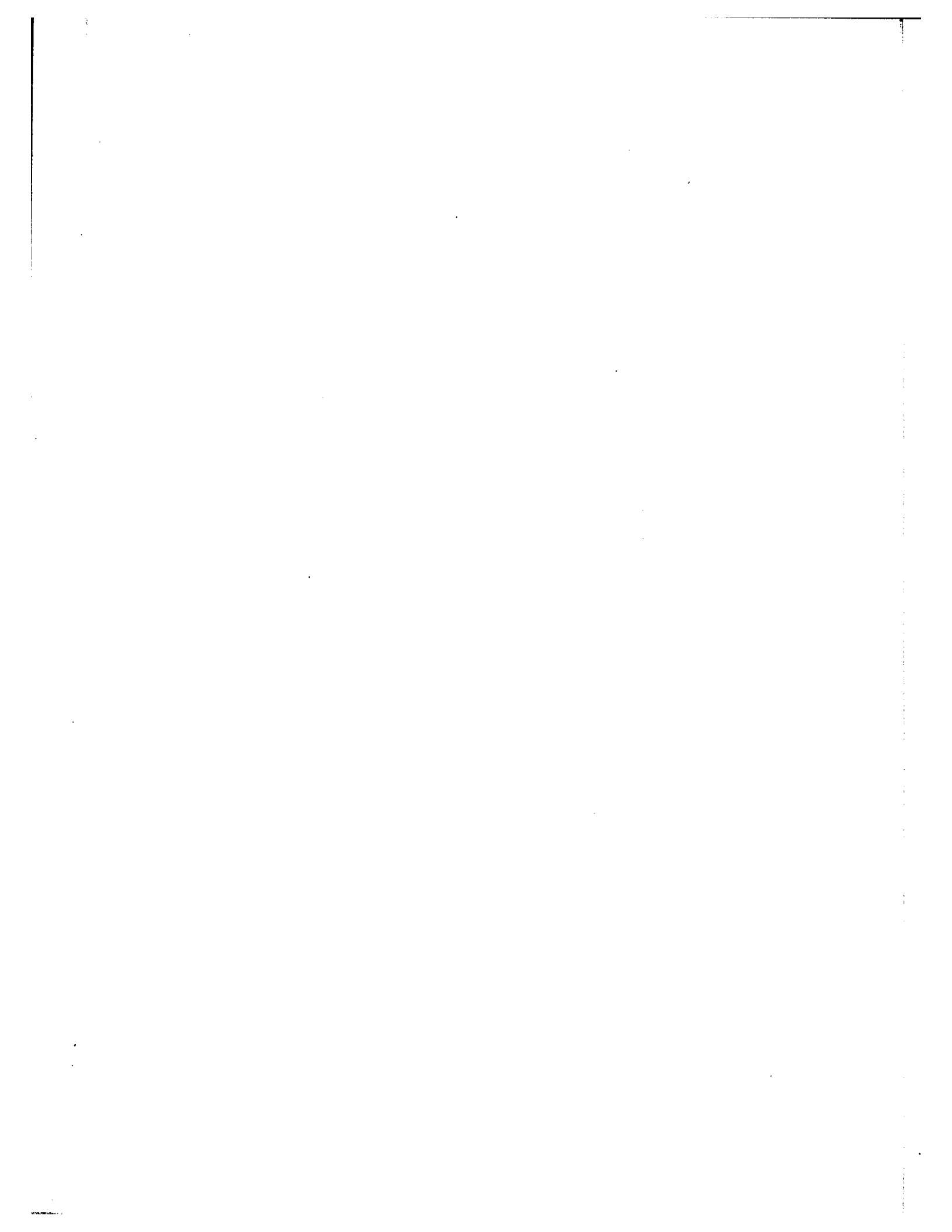
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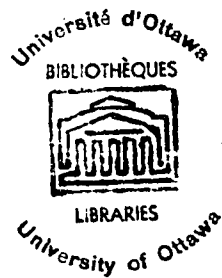
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ASCORBIC ACID - THYROXINE INTERRELATIONSHIP
IN COLD ACCLIMATION.

by

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Submitted in partial Fulfillment for the
degree of Doctor of Philosophy

in Biology,

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August 15, 1961.

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ABSTRACT

Experiments were designed to find whether ascorbic acid administration had any effect on the following parameters of metabolic alteration in cold-exposed animals: oxygen consumption, thyroxine secretory rate (TSR) and biosynthesis of ascorbic acid.

The results obtained may be summarized as follows:

1. No effect of ascorbic acid on the metabolic rate could be detected.
2. In untreated animals the TSR is increased during the first week of cold exposure, returns to normal in the following weeks and rises again after ten weeks. In the treated animals the TSR is also increased during the first week of cold exposure, but significantly less so than in the untreated animals. No effect of ascorbic acid is observable at room temperature.
3. The rate of biosynthesis of ascorbic acid is not altered significantly during the first three weeks of cold exposure, neither in the treated nor in the untreated animals. After six weeks of continuous exposure to cold, the rate of biosynthesis is significantly increased in untreated animals; this increase is prevented by the administration of ascorbic acid.

These results are discussed along with the current literature and it is concluded that the effect of ascorbic acid in cold exposed animals could be partly explained by a lowering

iii.

of the requirements for thyroid hormones. Cellular mechanisms of this action are briefly discussed and avenues of future research pointed out.

STATEMENT OF THE PROBLEM

The importance of the thyroid hormones in energy metabolism and hence in cold acclimation is now well established (Hart, 1958; Potter, 1958; Ingle, 1958). Also recognized is the fact that ascorbic acid administration has beneficial effects in animals exposed to cold (Dugal, 1952; DesMarais, 1957).

Since ascorbic acid is known to participate in the transport of electrons (Hoijer, 1960) while thyroxine has been shown to affect oxidative phosphorylation (Smith, 1960a and b; Beyer, 1960), it appears legitimate to assume that the beneficial effects of ascorbic acid administration in cold-exposed animals could be exerted through the thyroid hormones. Some evidence supporting this hypothesis has been obtained by DesMarais and recently reviewed (DesMarais, 1960). In order to further verify this hypothesis, it was decided to see whether ascorbic acid administration had any effect on oxygen consumption, thyroxine secretory rate and biosynthesis of ascorbic acid in animals either exposed to cold or kept at room temperature.

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PART ONE

REVIEW OF THE LITERATURE

Exposure of a homeotherm to cold environment produces a physiological strain throwing the homeostatic mechanisms of the animal out of equilibrium, especially during the initial period of cold exposure. Readjustments of various functions are brought into play for the maintenance of thermal balance. This brief review shall be limited to the role of the thyroid hormones and ascorbic acid in acclimation to cold.

I. The thyroid hormones in cold acclimation.

The increase in metabolic rate leading to cold-acclimation has been observed in several animal species. The various aspects of this metabolic adaptation have been extensively reviewed (Hart, 1958 and 1960).

The elevation of the BMR observed in cold-exposed animals may be regarded as one aspect of this increase, and since one of the fundamental actions of the thyroid hormones is the stimulation of the metabolic rate, it is not surprising that they have been so intimately linked to the process of cold acclimation.

Total thyroidectomy lowers the BMR by 40 % and consequently reduces the body temperature (Spence, 1959). The intravenous injection of a single dose of triiodothyronine increases

the BMR after a latent period of six to eight hours, which is less than that observed for thyroxine (Thibault, 1957). This substantiates the assumption that the thyroid hormones regulate the BMR although they cannot be considered as the sole agents of this regulation, since other substances, like the catecholamines, have been shown to increase the BMR in the presence of a fixed amount of thyroid hormones (Thibault, 1949). The role of the thyroid hormones in the extra-heat production of cold-exposed animals has been recently reviewed (Carlson, 1960; Cottle, 1960). Thyroid hyperactivity appears to be essential during the establishment of cold acclimation and less so after the new metabolic state has been attained when a normal level of circulatory thyroid hormones seems to exert a permissive effect on the calorogenic action of the catecholamines.

The mode of action of the thyroid hormones on the release of energy at the cellular level has been reviewed and is believed to be related to its uncoupling effect on oxidative phosphorylation (Smith, 1960a and b). An alternate role of the thyroid hormones has been suggested: the synthesis of enzymes activating the TPIII-regulated calorogenic shunt (Potter, 1958).

II. Ascorbic acid in cold acclimation.

Adequate amounts of vitamins were found to be essential for resistance to cold, but excessive quantities had no additional effects, except for ascorbic acid (Glickman, Keeton, Mitchell and Fahnestock, 1946).

In animals, the beneficial effects of ascorbic acid administration have been established by Dugal and co-workers. The several aspects of these effects have been extensively reviewed (Dugal, 1952; Deslaurais, 1957) and shall be briefly summarized. Successful acclimation to cold is accompanied by increased tissue levels of ascorbic acid, in both the rat and the guinea pig. Administration of this substance to rats and guinea pigs enhances their acclimation (growth) and resistance (survival) to cold. In cold-exposed animals given ascorbic acid, a large amount of this substance is retained in their tissues and various signs of stress are suppressed, like the hypertrophy of the adrenals in rats and guinea pigs, while others are ameliorated, like the occurrence of frost-bite in the monkey. These authors also suggest that the synthesis of ascorbic acid is increased in cold-exposed rats, since tissue levels of this substance are increased despite augmentation of the urinary excretion.

The beneficial effects of ascorbic acid administration have also been observed on some parameters of cold acclimation in man (LeBlanc, Stewart, Harrier and Whillans, 1954).

III. The mode of action of ascorbic acid.

The importance of both ascorbic acid and thyroid hormones in cold acclimation is suggestive of a relationship between these substances. This possibility is emphasized by the fact that many interactions between ascorbic acid and the thyroid hormones have been observed.

The administration of thyroid hormones or TSH results in a reduction of the ascorbic acid content of the liver, adrenals, thymus and kidneys (Slinnerlan and Momanzaki, 1959; Sure and Theis, 1938). Conversely, a rise in adrenal ascorbic acid has been observed in thyroidectomized animals (Paal and Brecht, 1937). It has also been found that the liver can store ascorbic acid when large doses of this substance are administered along with thyroxine or TSH (Crabtree and Trokojus, 1946). Since thyroxine administration prevents both the inactivation of ascorbic acid oxidase and the destruction of ascorbic acid (Benhamu, Magee and Dawson, 1959; Gemmill, 1951; Gemmill and Flunkett, 1952), adequate levels of thyroid hormones appear important for the maintenance of the active form of ascorbic acid, monodehydro-ascorbic acid. Ascorbic acid is also important in the metabolism of tyrosine and phenylalanine, acting as a co-enzyme for their oxidation (Gemmill, 1951). In the rat (Rangasaker and Dugal, 1958), blood plasma levels of these substances were reduced when the

animals were subjected to cold or given ascorbic acid; however, cold plus ascorbic acid lowered the plasma levels below that of either condition alone. This suggests a more rapid utilization of these metabolites and might have some importance in cold acclimation when one considers that tyrosine is the immediate precursor of the thyroid hormones while both substances are related to the medullary catecholamines.

In cold exposed animals, ascorbic acid administration has been shown, through histological evidence, to reduce the activation of the thyroid and to enhance the effects of low and inefficient doses of thyroxine (Deslauris, 1960). These findings suggest that the beneficial effects of ascorbic acid in cold-exposed animals could be mediated through the thyroid hormones. Other aspects of the function of ascorbic acid in cold acclimation have been reviewed (Hoijer, 1960) and shall be dealt with in the general discussion of these experiments.

PART TWO

EXPERIMENTAL

I. Description of methods

1. Animals, diet and treatments.

Male albino rats of the Wistar strain weighing between 110 and 140 grams were caged individually in metal cages with wire screen bottoms. They were fed Purina Lab Chow and tap water ad libitum. The animals on ascorbic acid treatment were given daily 150 mg of sodium ascorbate (Redoxon) through a feeding tube directly introduced into the stomach; this treatment was always started six days before the beginning of the experimental period; that is before the first day of week zero. After thyroidectomy, the operated animals were given a 1% solution of calcium lactate in the drinking water.

2. Temperatures of exposure.

The control animals were kept in the general animal quarters at a 'room temperature' varying between 20 and 26° C., while the others were exposed in a refrigerated room to a temperature of 2 ± 1° C.

3. Oxygen consumption.

The method of measurement involves the use of the Pauling oxygen analyzer (Model C 2) in an open circuit (Depocas

and Hart, 1957). In this technique, oxygen consumption is measured through determination of partial pressure changes in a metered portion of the dry, CO₂-free air leaving the animal cage (glass jar), the values obtained being unaltered by the respiratory quotient. The oxygen consumption measured was either the BMR obtained at 30° C. from rats fasted for 24 hours, or the actual metabolic rate determined at 30° (MR³⁰) or at 2° (MR²) according to the temperature of the water bath in which the glass jar was immersed. Oxygen consumption is expressed in ml. / hr. / B.W.^{0.5}.

4. Thyroxine secretory rate.

The thyroxine secretory rate (TSR) was obtained by thyroxine inhibition of the thyroïdal I¹³¹ release (Reineke and Singh, 1955), based on the assumption that this inhibition is proportional to the dose of administered thyroxine (Perry, 1951).

Each rat was injected intraperitoneally with 10 microcuries of carrier-free I¹³¹ (200 mc/mg) and 72 hours were allowed for fixation of the iodine by the thyroid and urinary elimination of excess isotope. External thyroid counts were determined 24, 48 and 72 hours after iodine administration and at appropriate intervals thereafter by the use of a scintillation detector (Nuclear Chicago, Model DS5-1) with the flat field collimator fitted with a half-inch thick lead plate in which a central

hole of three-quarter of an inch had been bored. Counts were recorded on a count rate meter (Nuclear Chicago, Model 181 A) with a 2% error level.

During counting, the animal was kept under light ether anaesthesia and the thyroid region centered over the opening in the lead plate for two consecutive one-minute counts, the average of which was used as the gross thyroid count. Counts were then recorded over the epigastric region and the gross thyroid count was corrected by subtracting half the body count plus the room background (Wolf, 1951), readjusting for isotopic decay. Thyroxine was injected subcutaneously every day with increase in dosage every 48 hours starting from 0.5 microgram per hundred grams of body weight, the thyroid counts being determined before each increase in dosage until the last count was between 80 and 100 per cent of the preceding one. A regression line was fitted by the method of least squares, resulting in a prediction equation: $Y = a + bX$ where Y is the percentage of the previous count and X represents the dosage of thyroxine at any interval. The thyroxine dosage is extrapolated to Y = 100 per cent and the result expressed in micrograms of l-thyroxine per day per 100 grams of body weight.

5. Biosynthesis of ascorbic acid.

The enzyme which converts l-gulonolactone to l-ascorbate

(Enzyme III) was assayed by the method of Grollman and Lehninger (1957) in the liver, which is the preferential site of ascorbic acid synthesis in the rat (Burns, 1959). L-gulonolactone was substituted for l-gulonate as used in the original method, since it saves one step in the synthesis of ascorbate (Chatterjee, Chatterjee, Ghosh, Ghosh and Guha, 1960).

Three aliquots of a one-third liver homogenate in 0.15 M. KCl were used for each determination. One was incubated for two hours at 37° C. with the precursor, a second incubated for the same time without the precursor and the third, also without precursor, was used for the determination of the zero time level of ascorbic acid in the liver being assayed. The amount of ascorbate formed is equal to the amount found in the first aliquot minus the amount found at zero time, while the amount found in the second aliquot, incubated without precursor, serves to determine the rate of destruction of ascorbate in the liver by reference to the zero time level. No correction is made for destruction rate when evaluating the biosynthesis, since the kinetics of these reactions has not been determined; one has to be satisfied if, under the conditions of measurement, the rate of destruction remains the same.

In all cases the 'total ascorbic acid', that is diketo-gulonate, ascorbate and dehydroascorbate, was determined by

the dinitrophenylhydrazine technique (Roe and Keuther, 1943), using the Coleman Junior Spectrophotometer (Model 6A) and freshly prepared standard solutions of l-ascorbate at suitable concentrations. The results were expressed in comparable units of enzyme activity, namely micromoles of total ascorbic acid formed per gram of wet liver per hour at 37° C.

6. Statistical analysis.

Most of the data have been subjected to the analysis of variance and whenever the F value was found to be significant, the multiple range test (Duncan, 1955) was used to locate the significant differences. This last test is also described and evaluated in 'Principles and Procedures of Statistics' (Steel and Torrie, 1960). The t test was also used whenever suitable to the experimental design.

There was no published statistical method for assessing the significance of the difference between two extrapolated values as determined in the TSR measurements. A method was thus devised by Dr. N.T. Gridgeman, statistician at the National Research Council of Canada. The method is based on the variance of both the observed and the calculated Y values and permits to calculate the standard error of the difference between two extrapolated X values, from which the significance is determined through the t test.

II. Results and discussion

A. Oxygen consumption.

1. Protocol.

The animals of the various groups, either treated or untreated with ascorbic acid, had their initial oxygen consumption measured at room temperature at week zero of the experimental period. Following that, oxygen consumption was measured weekly for five weeks either at room temperature or in the cold. The sixth week was used for determination of the TSR, after which the rats were thyroidectomized, those previously exposed to cold being returned to the refrigerated room 24 hours after the operation. They were then injected with a dose of L-thyroxine corresponding to the result of the TSR measurement and administered subcutaneously every day for seven days. At the end of this seventh week, oxygen consumption was determined and thyroxine administration suppressed; two more readings of oxygen consumption were obtained at eight and nine weeks of the experimental period.

In one particular experiment, the BIR of rats previously exposed to cold for six weeks was determined one, two and five hours after their return to room temperature. The animals were then returned to the cold room and their TSR was measured four weeks later, that is after ten weeks of exposure to cold.

2. Results.

a) Effect of ascorbic acid on oxygen consumption.

The results of these experiments are shown in table 1, page 13; the number of animals in each group is given in parenthesis and each average is followed by its standard error. The results of the Duncan's test are shown in table 2, page 14.

No effect of ascorbic acid administration was detectable in any of the experiments. It can also be observed that only the $\dot{V}O_2$ did increase significantly in cold exposed animals.

b) Effect of thyroidectomy.

After thyroidectomy and daily thyroxine administration for one week (week 7, table 1), the oxygen consumption is generally maintained at the previous level (week 5). Suppression of thyroxine therapy is followed by various degrees of diminution in oxygen consumption, without any significant difference from previous levels.

It is interesting to note, however, that at room temperature the fall in oxygen consumption is greater in untreated animals, while in the cold the treated rats exhibit the larger decrease. Whether this reversal is merely fortuitous as statistical analysis tends to show, or whether it represents a biologically significant phenomenon worthy of investigation could only be determined by further experimentation.

Table 1. Effect of ascorbic acid (AA) on Oxygen consumption.

Time in weeks	Intact animals										Thyroidectomized			Analysis of variance
											+ T ₄		Without T ₄	
	0	1	2	3	4	5	6	7	8	9	F	P		
B.M.R. 30	-AA (6)	25.0	28.7	23.6	23.9	25.4	24.5	TSR	27.1	20.9	16.6	4.48	<0.01	
		± 2.11	± 1.68	± 1.49	± 1.43	± 1.18	± 1.04		± 2.36	± 1.72	± 1.42			
	+AA (6)	27.9	28.7	24.5	25.4	28.2	26.1	TSR	22.0	19.8	21.0	5.27	<0.01	
		± 1.93	± 1.19	± 0.23	± 1.43	± 1.87	± 1.36		± 1.58	± 2.84	± 0.05			
M.R. 30	-AA (5)	26.1	24.6	23.7	32.2	29.0	30.4	TSR	27.0	22.4	21.6	3.81	<0.01	
		± 0.13	± 3.30	± 3.35	± 2.44	± 1.44	± 1.25		± 1.95	± 1.24	± 1.71			
	+AA (5)	25.9	24.7	27.7	28.3	23.4	25.4	TSR	25.3	20.7	22.7	1.53	>0.05	
		± 1.29	± 2.49	± 2.66	± 2.41	± 1.41	± 1.29		± 0.17	± 1.20	± 1.65			
B.H.R. 30	-AA (12)	20.2	22.2	25.8	26.5	26.1	27.5	TSR	29.4	25.6	23.3	12.67	<0.01	
		± 1.29	± 0.29	± 1.03	± 0.25	± 1.40	± 0.31		± 1.69	± 1.50	± 1.62			
	+AA (10)	19.7	23.7	28.4	26.3	26.6	27.1	TSR	29.9	25.5	21.0	5.06	<0.01	
		± 1.42	± 0.24	± 1.43	± 1.12	± 1.04	± 0.27		± 2.44	± 0.30	± 2.52			
M.R. 2	-AA (6)	26.7	--	36.6	31.9	30.9	33.0	TSR	32.4	28.8	30.4	1.02	>0.05	
		± 0.21		± 1.13	± 3.09	± 1.20	± 2.20		± 1.01	± 2.49	± 3.80			
	+AA (6)	25.6	--	31.7	34.1	30.8	33.6	TSR	30.2	25.3	20.6	4.50	<0.01	
		± 1.56		± 1.24	± 2.02	± 1.12	± 2.61		± 3.01	± 0.03	± 3.98			
M.R. 2	-AA (15)	22.6	45.4	49.6	51.3	48.0	48.0	--	--	--	--	64.63	<0.01	
		± 0.16	± 1.24	± 1.94	± 1.66	± 0.23	± 1.36							
	+AA (15)	23.9	45.0	46.9	50.8	49.6	49.8	--	--	--	--	47.78	<0.01	
		± 0.25	± 1.19	± 1.98	± 1.47	± 1.07	± 1.73							

Table 2. Oxygen consumption - Duncan's test of significance

Room temperature.

BMR	-AA	Weeks:	9	8	2	3	5	0	4	7	1
		Means:	<u>16.59</u>	<u>20.87</u>	<u>23.57</u>	<u>23.93</u>	<u>24.97</u>	<u>25.05</u>	<u>25.37</u>	<u>27.06</u>	<u>28.70</u>
	+AA	Weeks:	8	9	7	2	3	5	0	4	1
		Means:	<u>19.84</u>	<u>21.05</u>	<u>22.03</u>	<u>24.47</u>	<u>25.36</u>	<u>26.06</u>	<u>27.95</u>	<u>28.23</u>	<u>28.68</u>
MR ³⁰	-AA	Weeks:	9	8	1	0	7	2	4	5	3
		Means:	<u>21.59</u>	<u>22.39</u>	<u>24.63</u>	<u>26.09</u>	<u>27.02</u>	<u>28.69</u>	<u>29.03</u>	<u>30.41</u>	<u>32.22</u>

Cold room.

BMR	-AA	Weeks:	0	1	9	8	2	4	3	5	7
		Means:	<u>20.21</u>	<u>22.23</u>	<u>23.38</u>	<u>25.63</u>	<u>25.78</u>	<u>26.10</u>	<u>26.54</u>	<u>27.54</u>	<u>29.95</u>
	+AA	Weeks:	0	9	1	8	3	4	5	2	7
		Means:	<u>19.70</u>	<u>21.04</u>	<u>23.67</u>	<u>25.46</u>	<u>26.35</u>	<u>26.64</u>	<u>27.10</u>	<u>28.45</u>	<u>29.93</u>
MR ³⁰	+AA	Weeks:	9	8	0	7	4	2	5	3	
		Means:	<u>20.56</u>	<u>25.27</u>	<u>25.60</u>	<u>30.25</u>	<u>30.84</u>	<u>31.69</u>	<u>33.60</u>	<u>34.10</u>	
MR ²	-AA	Weeks:	0	1	4	2	5	3			
		Means:	<u>22.61</u>	<u>45.88</u>	<u>48.00</u>	<u>48.04</u>	<u>49.65</u>	<u>51.34</u>			
	+AA	Weeks:	0	1	2	4	5	3			
		Means:	<u>23.89</u>	<u>44.96</u>	<u>46.89</u>	<u>49.56</u>	<u>49.77</u>	<u>50.83</u>			

c) Effect of ascorbic acid on BMR.

Ascorbic acid administration is also without effect on the decrease of the BMR which occurs when cold-acclimated rats are returned to room temperature (table 3). It is seen that the BMR is back to room temperature level within five hours

following the return of the animals to room temperature.

Table 3. Effect of ascorbic acid on the BMR

Initial reading at room temperature	Time after return to room temp.			Analysis of variance	
	1st Hr.	2nd Hr.	5th Hr.	F	P
-AA 19.21±1.32 (6)	31.0±0.23	24.1±0.31	20.7±1.54	22.32	<0.01
+AA 20.1±1.64 (6)	32.1±1.99	27.2±2.16	21.6±1.68	9.42	<0.01

Duncan's test:

-AA	<u>19.2</u>	<u>20.7</u>	<u>24.1</u>	<u>31.0</u>
+AA	<u>20.1</u>	<u>21.6</u>	<u>27.2</u>	<u>32.1</u>

d) Seasonal changes in oxygen consumption.

It happened, for various reasons, that most of the experiments were performed during definite periods of the year: in winter, from mid-january to the end of march, and in summer from mid-june to the end of august. Oxygen consumptions of the various groups have thus been compared on this basis, using the zero week determination of initial oxygen consumption at room temperature, including the MR² groups which had their initial oxygen consumption measured at 30°. The results of these comparisons can be found in table 4 (page 16). No effect of ascorbic acid treatment has been detected. Although the Duncan's test did not show any significant difference, it is nevertheless striking to see that the difference between summer and winter

oxygen consumptions is greater for the BMR than for the MR³⁰.

Table 4. Effect of season on oxygen consumption

	BMR		MR ³⁰	
	Summer	Winter	Summer	Winter
-AA	20.2±1.29 (12)	25.0±2.11 (6)	22.6±0.16 (15)	26.4±0.18 (11)
+AA	19.7±1.42 (10)	27.9±1.93 (6)	23.9±0.25 (15)	25.7±1.32 (11)

Analysis of variance: F = 29.32 - P = 0.001

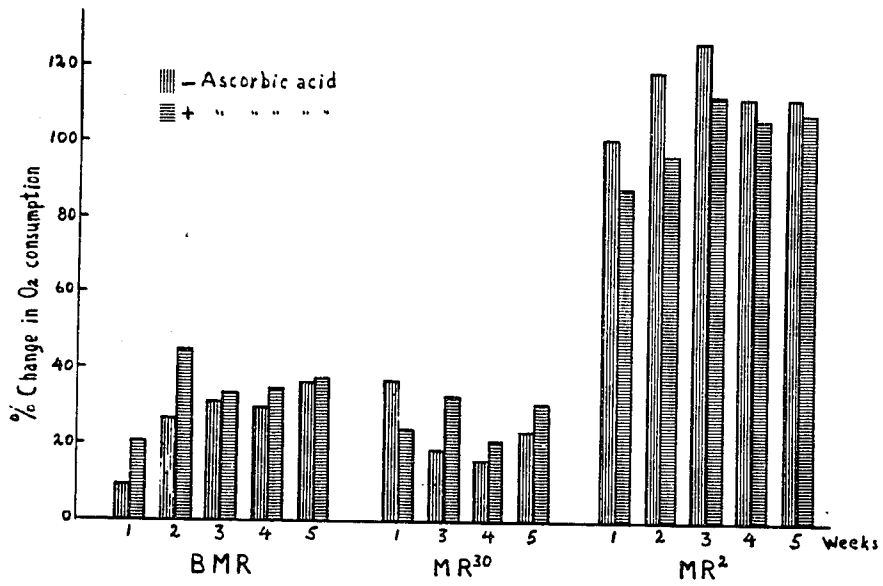
Duncan's test:

27.9 26.4 25.7 25.0 23.9 22.6 20.2 19.7

In parenthesis: number of animals.

3. Discussion and conclusions.

The results obtained show that the BMR and the MR³⁰ of rats exposed to cold are raised by almost the same value when the data are expressed as per cent increment over the initial reading at room temperature (see Histogram, page 17). Although the increase in MR³⁰ (average of all determinations in the cold) is slightly greater than that observed by Héroux, Depocas and Hart (1959), that is 23.9 % as compared to 17.1 %, it appears surprising that the augmentation of the BMR be as great as that of the MR³⁰. The combined initial values for the BMR and MR³⁰ of our rats subsequently exposed to cold were 19.9 and 26.2 respectively, as compared to 22.8 in the experiments re-



Histogram showing the percentage of change in metabolic rate as compared to room temperature (week 0).

ported by Héroux et al. These differences can be interpreted on the basis of discrepancies in experimental conditions. Our rats were maintained at the fluctuating temperature of the animals quarters and thus constantly submitted to mild thermal stimulation, while those of Héroux et al. had been kept at a constant temperature of 30° C., in the zone of thermal neutrality, and consequently avoiding this stimulation. This might explain why the MR^{30} obtained by these authors is closer to the BMR than to the MR^{30} observed in our experiments; consequently, our increase in MR^{30} upon exposure to cold is not greater than that of the BMR because of the wider difference at the start. Finally, since the temperature of acclimation was 2° in our experiments as compared to 6° in those of Héroux et al., it is not surprising that the increase in MR^{30} which we observed was greater than that found by these authors.

From the results obtained, it can be concluded that ascorbic acid administration has no effect on the metabolic rate of rats constantly exposed to low temperature. If, on the other hand, one considers the fact that ascorbic acid administration is without effect on the return of the BMR to room temperature levels when cold-acclimated animals are removed from the cold room, one must conclude that it does not affect the basal heat production of acclimated animals, not more than

it does affect the mechanisms responsible for extra-heat production in cold-exposed rats.

Finally, the observation that the difference between summer and winter values are greater for the BMR than for the MR³⁰, although not significantly so, would tend to show that the basal heat production may be modified by the season. Such differences, despite their lack of statistical significance, might affect the magnitude of the changes in metabolic rate upon exposure to cold since the starting point would not be the same; their existence should therefore be recognized.

B. Thyroxine secretory rate.

1. Protocol.

In both room temperature and cold exposed rats, the TSR was determined in the sixth week of the experimental period on the same animals which had been used to follow the changes in metabolic rate. The TSR after ten weeks of exposure to cold was obtained from the rats utilized for the study of the return of the BMR to room temperature level. Lastly, separate groups of animals were used for the determination of the TSR after one, two and three weeks of cold exposure.

2. Results.

The data obtained have been grouped in table 5, page 20. Since the individual regression curves for TSR are not essential

to the discussion, they have been grouped at the end of this section from page 27 to page 34.

Table 5. Effects of cold exposure and ascorbic acid (AA) administration on the thyroxine secretory rate.

Weeks in the cold	- AA	+ AA	Difference ± S. E.	t	p	Fig.
None	1.74 (5)	2.26 (5)	0.52 ± 0.39	1.33	N.S.	1
None	1.96 (6)	2.25 (6)	0.29 ± 0.27	1.07	N.S.	2
1	7.88 (6)*	4.79 (6)*	3.09 ± 0.33	9.45	<.01	3
2	3.77 (9)*	2.40 (9)	1.37 ± 1.54	0.89	N.S.	4
3	2.18 (6)	3.06 (6)	0.88 ± 1.79	0.49	N.S.	5
6	2.49 (9)	2.13 (9)	0.36 ± 1.02	0.35	N.S.	6
6	2.37 (5)	1.85 (5)	0.52 ± 0.83	0.63	N.S.	7
10	3.66 (5)*	2.71 (5)	0.45 ± 1.18	0.81	N.S.	8

In parenthesis: number of animals

* Significantly different ($P < 0.01$) from combined TSR values at room temperature.

In the untreated animals, the TSR is significantly increased over the room temperature level during the first and second weeks in the cold; it then returns to control levels to rise again significantly after ten weeks of cold exposure. In the treated animals, the TSR is increased during only the first week, but this augmentation is significantly lesser than that observed in the untreated animals for the same period.

3. Discussion and conclusion.

The significance of these results cannot be properly evaluated unless the technique used is entirely reliable. Since its original description (Reineke and Singh, 195~~6~~⁵), it has been used by different investigators in a variety of experimental conditions (Henneman, Reineke and Griffin, 1955; Lodge, Lewis and Reineke, 1957; Wada, Berswordt-Wallrabe and Turner, 1959; Pipes, Grossie and Turner, 1960; Premachandra and Turner, 1960; Anderson, Grossie and Turner, 1961; Himeno, Tanabe and Komiyama, 1961; Van Heyningen, 1961). They all seem to accept the validity of the technique, although Henneman et al. and Lodge et al. observed that an increased release of thyroidal iodine is obtained when doses of thyroxine larger than the calculated TSR value are administered, which they discard as indifferent to the validity of the technique. We also have observed this phenomenon. A likely explanation of this occurrence would be that the increased release is caused by the return of labelled thyroxine from the intra- and extra-vascular spaces of the thyroid into the general circulation to equilibrate with the excess of exogenous thyroxine introduced. This suggestion is supported by the finding that the specific activity of thyroxine in the plasma is increased following the administration of a load of non-labelled thyroxine in rats previously injected with radioiodine (Ingbar

and Freinkel, 1960). A more serious criticism may be voiced against the TSR technique following the observation by Héroux (personal communication) that comparable groups of similarly treated rats were found to have different TSR values according to the starting dosage level of thyroxine injected for the determination, the TSR being lower when the dose of thyroxine was lesser. This finding casts serious doubt on the very basic assumption of the technique: the proportional inhibition of iodine release by the administration of progressively increasing doses of thyroxine (Perry, 1951). We have undertaken to verify Héroux's observation; if it was confirmed, the validity of the technique would have to be reassessed, and it would show that, despite the enormous volume of publications on the internal physiology of the thyroid, it is far from being well understood.

In this perspective, our conclusions can only be accepted with prudent reserve. In untreated rats, the increase in thyroid function upon exposure to cold appears to be a biphasic phenomenon. How long is a higher secretory rate maintained after the tenth week has not been determined, but Héroux (personal communication) has found secretory rates of 6°-acclimated rats to be higher than those of 30°-acclimated ones three months after exposure to cold; however these findings can hardly be compared to ours, since rats acclimated to 30°, in the zone

of thermal neutrality, are likely to have a decreased thyroid function as compared to our controls kept at room temperature. In the treated animals, the TSR values observed appear to show that ascorbic acid administration decreases the need for thyroid hormones in cold-exposed animals, since the TSR is increased only during the first week and less so than in the untreated animals.

C. Biosynthesis of ascorbic acid.

1. Protocol.

The livers were removed at autopsy, immediately frozen on dry ice and kept in a deep-freeze refrigerator. Tests have shown that the enzyme activity survived storage of the whole tissue, homogenates or fractions in the frozen state (Grollman and Lehninger, 1957).

The animals used for the determinations were sampled at random from the groups used for MR^2 (six weeks of cold exposure) and from those used for TSR measurement after one, two and three weeks in the cold. The animals kept at room temperature were separate groups, and the treated ones had received ascorbic acid for six weeks before removal of the liver.

2. Results.

The results appear in table 6, page 24.

Although the change is not significant, both the treated and untreated rats showed a decrease in liver biosynthesis of

Table 6. Effects of cold exposure and ascorbic acid (AA) administration on the biosynthesis of ascorbic acid.

Weeks in the cold			Difference	t	p	
	- AA	+ AA	± S. E.			
None	13.18±0.73 (6)	12.78±0.63 (6)	0.40±0.96	0.42	N.S.	
1	5.97±0.11(10)	6.93±0.68(13)	0.96±0.69	1.25	N.S.	
2	10.31±2.02 (7)	9.37±1.48 (7)	0.94±2.50	0.38	N.S.	
3	10.84±0.98 (5)	12.70±0.33 (4)	1.86±1.03	1.80	N.S.	
6	20.53±2.59 (6)	13.56±0.77 (6)	6.97±2.70	2.58	<0.05	
Analysis of variance:			F	P		
	- AA		13.55	<0.01		
	+ AA		9.29	<0.01		
Duncan's test:						
	- AA	<u>5.97</u>	<u>10.31</u>	<u>10.84</u>	<u>13.18</u>	20.53
	+ AA	<u>6.93</u>	<u>9.37</u>	<u>12.70</u>	<u>12.78</u>	13.56

In parenthesis: number of animals.

ascorbic acid after one week of exposure to cold. After six weeks of cold exposure, the untreated animals exhibited a significant increase of Enzyme III activity, while it stayed at room temperature level in the treated ones exposed to cold for the same time. At room temperature, the animals treated for six weeks with ascorbic acid did not show any change in the biosynthesis of this substance.

3. Discussion and conclusion.

Before proceeding with the discussion, it is essential to remind the reader that the results obtained do not necessarily reflect actual reaction rates in the intact organ, but merely permit an appraisal of what should be expected in vivo.

It has been observed that tissue levels of ascorbic acid are increased in the organs of cold-exposed rats (Dugal and Thérien, 1947a) and guinea pigs (Dugal and Thérien, 1947a and b; Thérien and Dugal, 1947), while the urinary excretion of ascorbic acid is increased in the rat and decreased in the guinea pig (Thérien and Dugal, 1949). These observations suggest that exposure of rats to cold induces an increase in ascorbic acid synthesis with a consequent elevation of tissue levels despite the increased excretion, while the guinea pig increases its tissue levels by reducing the urinary loss of this substance.

These authors have observed that the increase in urinary excretion of ascorbic acid is immediate following exposure of rats to cold; since we have observed that, at this time, the rate of biosynthesis of this substance is not increased, some of it must be lost by the tissues. Later, that is some time between the third and the sixth week of cold-exposure, and certainly at the sixth week, the rate of biosynthesis is increased; this would account for the elevation of tissue

levels at that time.

Our results show that the daily administration of ascorbic acid does not modify the rate of its synthesis during the first three weeks of exposure to cold. It thus seems that the extra-vitamin given during this period provides the animal with an amount of this substance exceeding its capacity to obtain it from endogenous source. Since ascorbic acid has beneficial effects in these animals, it follows that it must be used somehow by the organism, thus emphasizing the physiological importance of early administration of this substance to cold-exposed animals.

The chronic administration of ascorbic acid has been found to prevent the increase in its biosynthesis after six weeks of exposure to cold. Since it has no such effect at room temperature, it would seem that, in cold-exposed animals, it either inhibits the mechanism activating the biosynthesis or suppresses the stimulus for its activation. The increased rate of biosynthesis in rats which have reached the state of acclimation could also mean that, at this stage, they would be less dependent upon an exogenous supply of this vitamin.

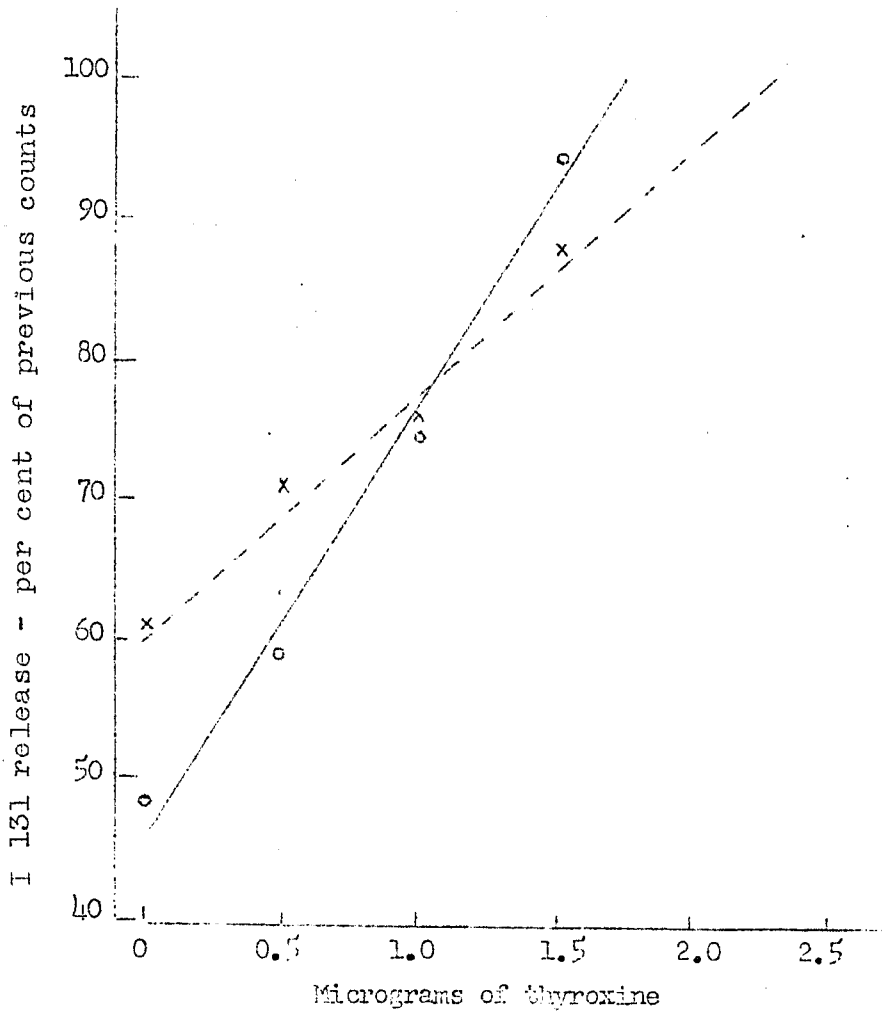


Figure 1. T5R at room temperature.
Ascorbic acid treated: x- - -x
Untreated: o——o

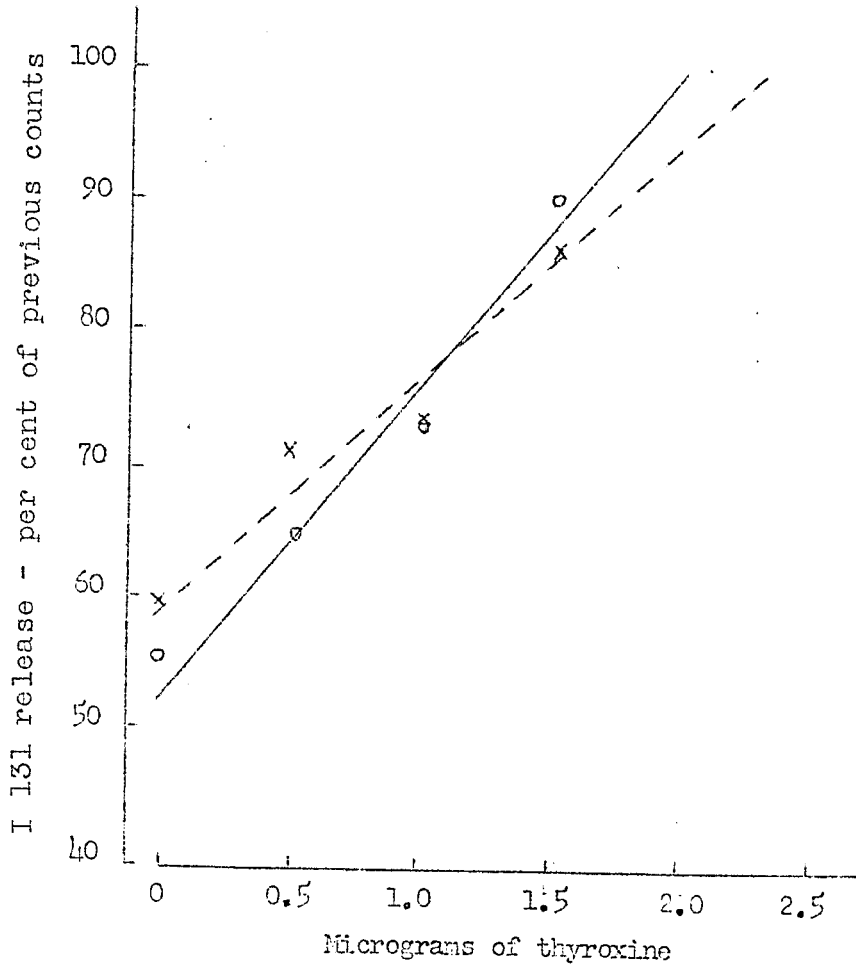


Figure 2. TSR at room temperature.
Ascorbic acid treated: x — — x
Untreated: o — — o

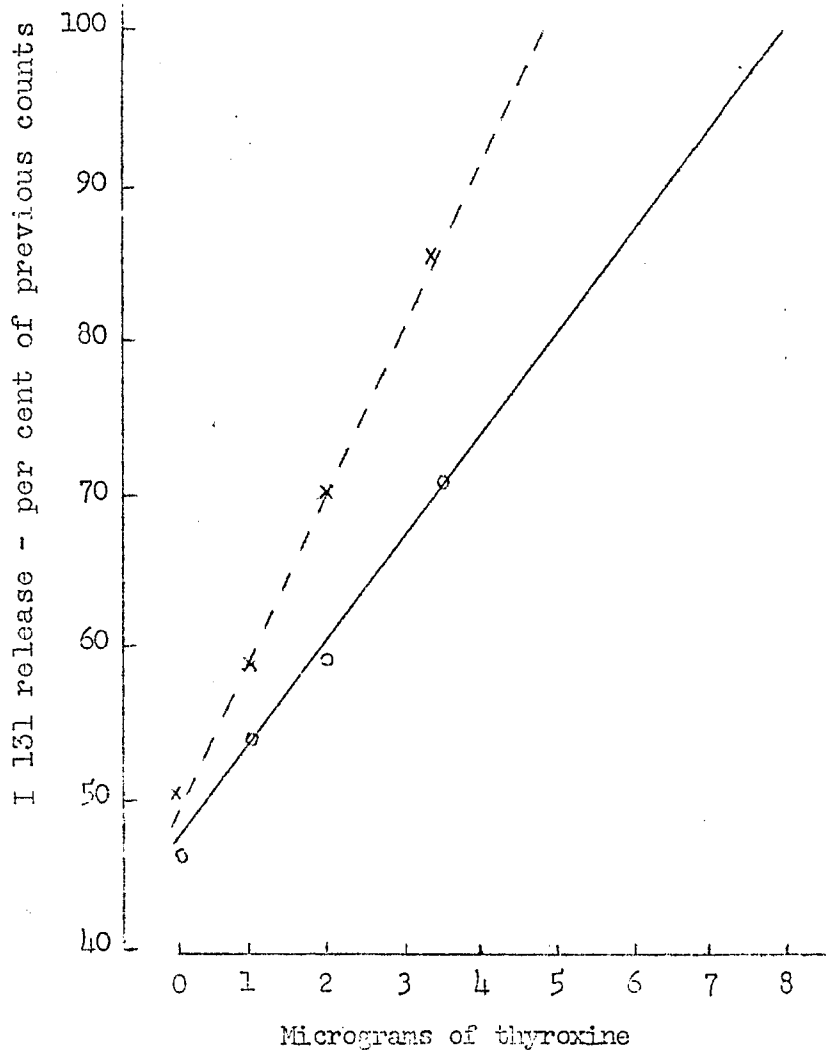


Figure 3. TSR after one week of exposure to cold
Ascorbic acid treated: x - - - x
Untreated: o - - - o

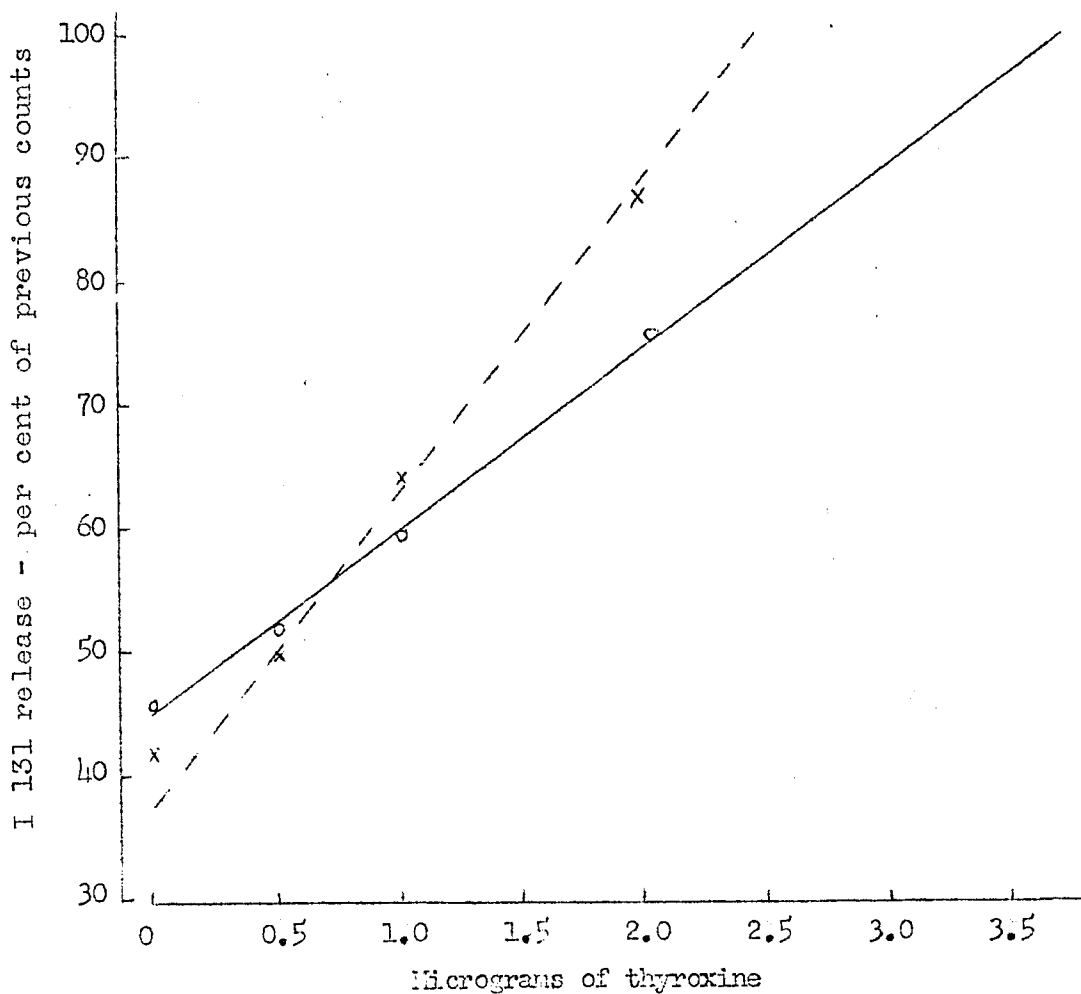


Figure 4. TSE after two weeks of exposure to cold
Ascorbic acid treated: x- - -x
Untreated: o——o

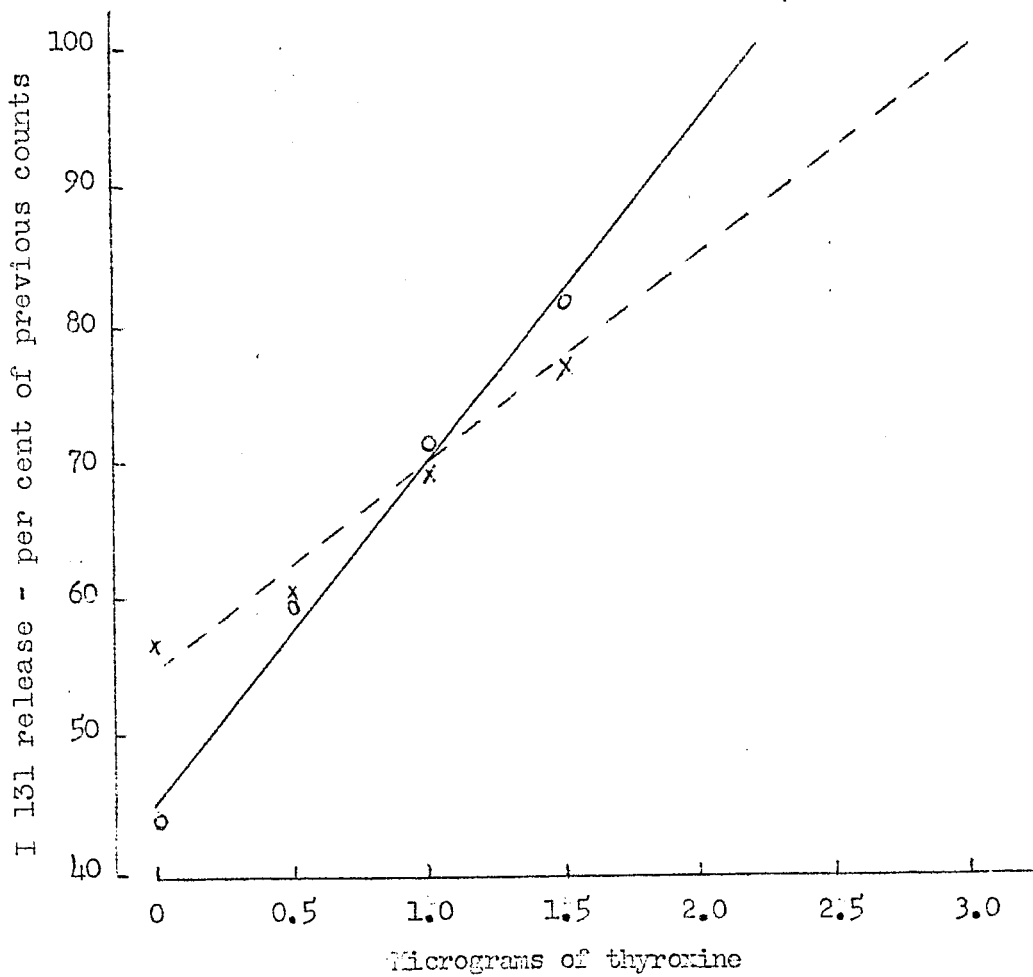


Figure 5. TSR after three weeks of exposure to cold
Ascorbic acid treated: x - - - x
Untreated: o ——— o

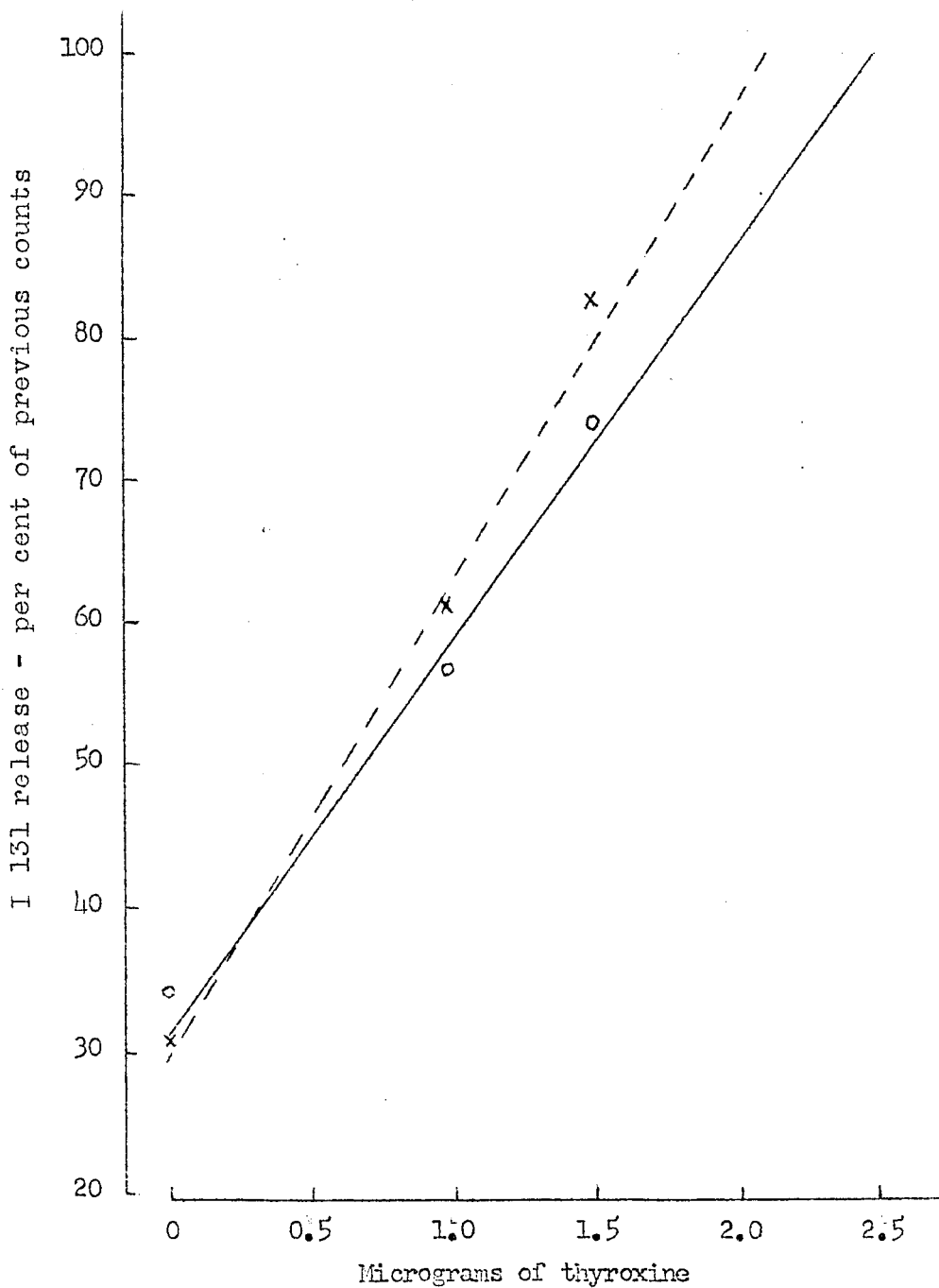


Figure 6. TSR after six weeks of exposure to cold
Ascorbic acid treated: x - - - x
Untreated: o - - - o

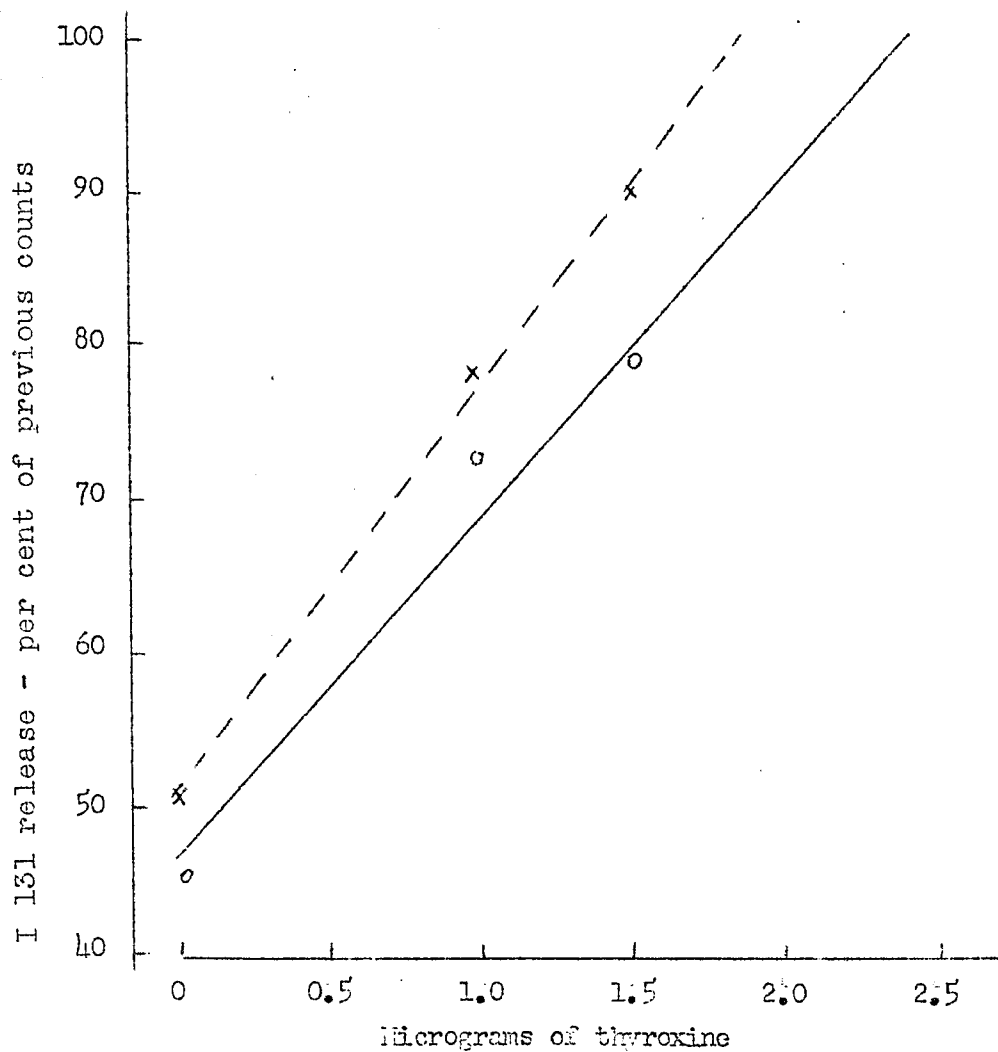


Figure 7. TSR after six weeks of exposure to cold
Ascorbic acid treated: x - - - x
Untreated: o - - - o

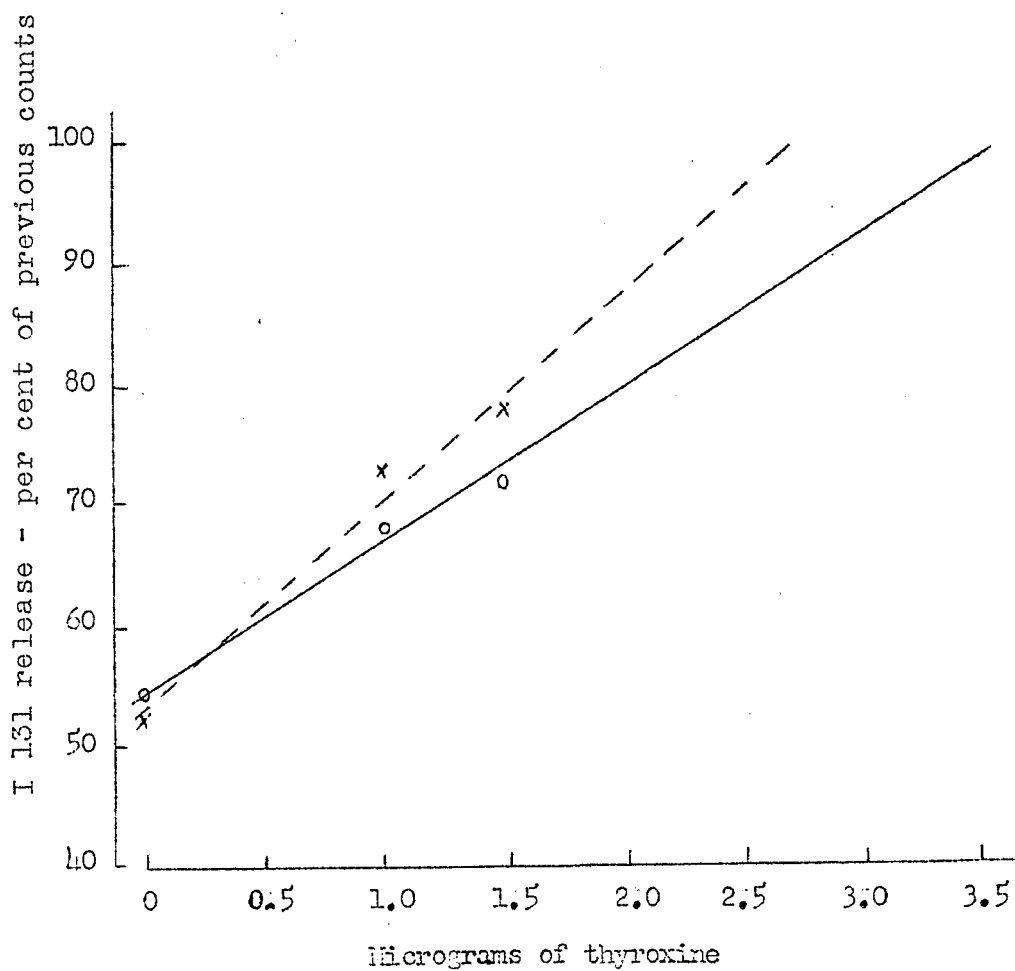


Figure 8. TSR after ten weeks of exposure to cold
Ascorbic acid treated: x- - -x
Untreated: o ——— o

PART THREE

General discussion and conclusions

The results obtained in these experiments provide evidence that ascorbic acid administration does not affect the changes in metabolic rate induced by exposure of the rat to cold. The increase in metabolic rate being the most important feature of cold acclimation (Hart, 1958 and 1960), one of the possible explanations for the beneficial effects of ascorbic acid administration to cold-exposed animals was a direct influence on the level of heat production. Since the energy metabolism was the same in both treated and untreated animals, the effects of ascorbic acid must be sought at the level of the mechanisms responsible for increased heat production. Such effects could be exerted indirectly through the thyroid hormones or directly by some vicarious action. In either case the need for thyroid hormones would be reduced.

Administration of ascorbic acid has been shown to decrease the stimulation of the thyroid gland in both the rat (DesMarais, 1955) and the guinea pig (DesMarais and Gagnon, 1955) exposed to cold. Since the administration of ascorbic acid was without effect on the sensitivity of the thyroid gland to thyrotrophic hormone (DesMarais, 1958), its influ-

ence on the thyroid gland was believed to be exerted through the feed-back mechanism or the blood level of thyroid hormones. This was indirectly supported by the finding that the need for thyroid hormones was decreased in thyroidectomized animals given ascorbic acid, at least during the first week of cold exposure (DesMarais, 1956). With all due reserve as to the validity of the technique used, it is comforting to note that this observation of DesMarais is confirmed by our finding that the increased in TSR observed during the first week is significantly lessened by ascorbic acid administration. Thus the previous assumption that the inhibiting action of ascorbic acid on the stimulation of the thyroid by cold exposure was due to a decreased level of circulating thyroid hormones appears to be correct. Ascorbic acid would thus act at the peripheral level either through enhancement of the activity of the thyroid hormones on the release of energy or by a direct action on a competitive process of energy liberation. Evidence exists for the two modes of action.

Thyroxine and its analogues have been shown to uncouple oxidative phosphorylation (Solomon and Dowling, 1960), producing this effect by altering the permeability of the mitochondrial membrane (Dickens and Salmony, 1956; Maruyama and Kobayashi, 1956). The uncoupling of oxidative phosphorylation by the thyroid hormones decreases the efficiency of the syn-

thesis of ATP with a consequent loss of energy as heat, energy which would otherwise have been stored by the organism. The role and importance of this mechanism in cold acclimation has been emphasized by Smith (1960 a and b). On the other hand, monodehydroascorbic acid, the active form of ascorbic acid, favors the transport of electrons through the cytochrome system without increasing phosphorylation (Cooperstein, 1959; Kielly and Bronk, 1957). It would be of interest to see whether or not this effect of ascorbic acid is exerted through the thyroid hormones. If such, it would explain why less thyroid hormones are needed by cold-exposed rats given ascorbic acid to produce the same amount of heat as the untreated ones.

The possibility of ascorbic acid having a direct effect on oxygen activation and hence on energy release has also been demonstrated. Recent studies (Staudinger, Krisch and Leonhauser, 1961; Strittmatter and Velick, 1956) have actually demonstrated a flavoprotein-catalyzed electron transport from DPNH to oxygen only in the presence of catalytic amounts of ascorbic acid. Evidence for this mechanism, suggested by Kern in 1954, has been confirmed by direct measurement using electron resonance spectroscopy (Yamanzaki, Mason and Pitte, 1959). Dehydroascorbic acid was shown to act as the intermediate hydrogen acceptor in the system, since it is impossible for ascorbic acid to accept hydrogen in its reduced form.

These two mechanisms of ascorbic acid activity at the level of energy release, either direct or through the thyroid hormones, are not mutually exclusive; they would not appear to be additive either, since the total energy release (metabolic rate) is the same with and without ascorbic acid administration. It thus remains that they would be competitive, other factors being theoretically capable of favoring one or the other.

This discussion clearly outlines the direction of future research on the mode of action of ascorbic acid in cold-exposed animals. Several avenues are open to research and should require the diligence of many workers in various disciplines.

In conclusion, our experiments have provided evidence of a relationship, direct or indirect, between ascorbic acid and the thyroid hormones in cold-exposed animals.

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