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Effect of Erythropoietin on the Peroxidase and Tyrosine-Iodinase Activity of Mouse Thyroid and Submaxillary Gland

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During an attempt to explore the mechanism of cobalt and vitamin B_{12} effect on the peroxidase and tyrosine iodinase activity, it was observed that cobalt and not vitamin B_{12} *in vivo* elicits a high plasma titer of a humoral factor, erythropoietin, which caused increased ^{59}Fe incorporation into red blood corpuscles. This erythropoietin was extracted from plasma or kidney and partially purified. This partially purified erythropoietin preparation when injected into starved rat caused increased ^{59}Fe incorporation into red blood corpuscles. The potency of the partially purified erythropoietin preparation from plasma and kidney was compared. This partially purified erythropoietin, as well as sheep step-I erythropoietin, when administered to mice gave rise to a response almost similar to cobalt administration. It appears that the effect of cobalt on the peroxidase or tyrosine iodinase activity might be mediated through the elevation of the erythropoietin titer of plasma. Moreover, this erythropoietin was found to stimulate both peroxidase and tyrosine iodinase activity of the enzyme preparation of submaxillary gland in experiments *in vitro*. Erythropoietin, purified further in our laboratory, also produced the same effect. Neuraminidase-treated erythropoietin, in contrast, failed to execute any such stimulatory effect on the iodination of tyrosine.

Recently, it has been reported from our laboratory [1] that Co^{2+} or vitamin B_{12} *in vivo* stimulates the peroxidase activity of mouse thyroid and submaxillary glands. The tyrosine iodinating enzyme activity of both the glands is also stimulated by vitamin B_{12} *in vivo*. Studies *in vitro* indicated that only Co^{2+} has a direct inhibitory effect on the enzymatic incorporation of iodide by the thyroid gland while cobamide coenzyme has a direct stimulatory role on the same process of the submaxillary gland. It was suggested that the effect of Co^{2+} or vitamin B_{12} *in vivo* may be mediated, at least partially, through the cobamide coenzyme [1].

However, as these effects of Co^{2+} or vitamin B_{12} on these enzymes cannot be fully explained by mediation only through the cobamide coenzyme, some other mechanism was therefore looked for. Survey of the literature points out that Co^{2+} also stimulates erythropoiesis [2, 3]. It is now clear that this effect of Co^{2+} is mediated not through vitamin B_{12} coenzyme but through the elaboration of a high level of erythropoietin in plasma [4] which is involved in the differentiation of red blood corpuscles along with hemoglobin biosynthesis [5]. The same observation has also been confirmed demonstrating that Co^{2+} and not vitamin B_{12} resulted in the elevation of the level of plasma erythropoietin. The extraction and partial purification of this erythropoietin from plasma or kidney of cobalt-treated mice and studies of its potency have been presented. Finally the results on the effect of this partially purified erythropoietin as well as the erythropoietin from other sources on the iodinating enzyme system of mouse thyroid and submaxillary glands are reported in the present communication.

Enzymes. Peroxidase (EC 1.11.1.7); neuraminidase (EC 3.2.1.18).

MATERIALS AND METHODS

Male Swiss mice of average body weight 25 g were used throughout the experiments. $Na^{131}I$, $^{60}CoCl_2$ and $^{59}FeCl_3$ were obtained from Bhabha Atomic Research Centre, India. Sheep plasma step I erythropoietin (0.4 U/mg) was purchased from Connaught Medical Research Laboratory (University of Toronto, Canada) and human urinary erythropoietin (2.6 U/mg) was obtained through the courtesy of Dr J. M. Stengle (N.I.H., Bethesda). DEAE-cellulose and neuraminidase (type V) were procured from Sigma Chemical Co. Amidoschwarz and *n*-butanone were obtained from E. Merck. All other materials used were of reagent grade.

Assay of Erythropoietin by ^{59}Fe Incorporation Study

The erythropoietic activity of Co^{2+} or vitamin B_{12} was determined by studying the ^{59}Fe incorporation into red blood corpuscles. Co^{2+} (0.044 μg/g) or vitamin B_{12} (0.5 μg/g) was daily injected separately into a group of mice for six or three days respectively. On the last day of injection, 1.0 μCi of $^{59}FeCl_3$ in 0.2 ml 0.9% saline was injected intraperitoneally. Blood was then drawn at different time intervals after ^{59}Fe administration and collected in a test tube containing a few milligrams of sodium citrate as an anticoagulant. Radioactivity of an aliquot of this blood was measured in a solid scintillation γ-ray spectrometer. ^{59}Fe incorporation into red blood corpuscles was then calculated assuming the blood volume to be 7% body weight.

Erythropoietin, after partial purification from plasma or kidney, was assayed by studying the ^{59}Fe incorporation into red blood corpuscles of starved rat according to the method of Fried *et al.* [6]. Male rats weighing 100—150 g were used for the assay. Rats were starved for 30 h and grouped as control and experimental, each group containing at least three animals. The control group was injected subcutaneously with 0.5 ml of the erythropoietin preparation obtained from plasma or kidney of normal animals, while the experimental group received 0.5 ml of the same from plasma or kidney of Co^{2+}-treated mice. 16 h later, 1.0 μCi $^{59}FeCl_3$ in 0.9% saline (0.2 ml) was injected intraperitoneally to each animal and blood was collected from the heart 18 h after ^{59}Fe injection. 0.1 ml of the blood was then taken on a planchet, spread uniformly, dried in air and finally the radioactivity was measured in a solid scintillation γ-ray spectrometer. The percentage of ^{59}Fe incorporated into red blood corpuscles was calculated assuming the blood volume as 5% (and not 7% as in case of mouse).

Extraction of Erythropoietin from Plasma or Kidney

Co^{2+} at a dose of 0.044 to 0.1 μg/g body weight stimulates the peroxidase activity, the stimulation being maximum after six days of administration [1]. Hence approximately 2.0 μg Co^{2+} per mouse was injected intramuscularly for six days in a group of mice containing not less than six animals. 16 h after the last injection, they were ether-anaesthetised and blood was drawn from the heart and collected as before. The kidneys of each mouse were also taken out at the same time.

Extraction from Plasma

Erythropoietin was extracted from plasma according to the method of Borsook *et al.* [7]. The extraction was carried out in parallel from the blood of normal as well as Co^{2+}-treated mice. Blood (10 ml from six animals) was centrifuged at 2000 rev./min for 15 min and the plasma was collected. The plasma was acidified with 0.1 N HCl to bring down the pH to 5.5. This acidified plasma was then heated to 80 °C for 10 min and centrifuged to collect the supernatant. The coagulum was washed with distilled water, heated as before, centrifuged and the supernatants were combined. The process was repeated once more and finally the combined supernatant was dialysed overnight against one liter of distilled water to remove dialysable Co^{2+}, if any. The straw-coloured dialysed material was cloudy in appearance and the colour was more intense in the case of the plasma of Co^{2+}-treated mice. This was then lyophilised and the volume was finally made up to the original volume of the plasma with distilled water. This preparation was then assayed for erythropoietin as described earlier.

Extraction from Kidney

Preliminary extraction of erythropoietin from kidney was carried out according to the method of Contrera *et al.* [8]. The normal as well as Co^{2+}-treated kidneys were extracted simultaneously. The kidneys (3 g from six mice) were cut into pieces and homogenised in 6 ml 0.02 M hypotonic phosphate buffer pH 6.8 to get a 50% homogenate. The homogenate was then centrifuged at $37000 \times g$ for 30 min in a Sorvall RC-2B refrigerated centrifuge and the supernatant was collected. This supernatant was then dialysed overnight against one liter of 0.9% NaCl solution and assayed for erythropoietin by ^{59}Fe incorporation study as described before.

Potency of the Preparations

The potency of the partially purified erythropoietin was determined from a standard curve of ^{59}Fe incorporation (Fig. 1) using sheep erythropoietin as a standard. The assay procedure was the same as already described. The potency was expressed as U/mg protein.

Effect of Erythropoietin on the Peroxidase and Tyrosine-Iodinase Activity

For the assay of peroxidase and tyrosine iodinating enzyme activity of mouse thyroid and submaxillary glands, 0.4 unit of the extracted erythropoietin preparation or sheep erythropoietin was daily injected subcutaneously for six days. 16 h after the last injection, the animals were sacrificed and the enzyme was prepared from the thyroid or submaxillary gland and assayed as described earlier [1]. Peroxidase activity was also assayed by measuring the formation of triiodide [9]. The incubation mixture contained in a final volume of 3 ml: 150 μmol acetate buffer pH 5.0 for thyroid and 4.6 for submaxillary enzyme

preparation, 1 μmol KI, 2 μmol H_2O_2 for thyroid and 500 nmol for submaxillary enzyme, enzyme preparation of a suitable volume and water. The increase of absorbance was measured for 1 min at an interval of 10 s at 353 nm to measure the triiodide (I_3^-) formation. For study *in vitro*, sheep or human erythropoietin was added in the incubation mixture as described in the legends of figures or tables.

Purification of Erythropoietin on DEAE-Cellulose

Sheep erythropoietin (step I), purchased from Connaught Medical Research Laboratory was further purified according to the method of White *et al.* [10]. Erythropoietin, equilibrated against 0.0375 M NaCl pH 4.5, was applied on a DEAE-cellulose column (9 cm × 1 cm) equilibrated with the above-mentioned solution. Gradient elution was carried out using 1.0 M NaCl in the reservoir and 100 ml 0.025 M NaCl in the mixing chamber. Fractions of 3.0 ml were collected in each tube.

Protein Estimation

Protein was determined according to the method of Lowry *et al.* [11] using bovine serum albumin as standard.

RESULTS

The role of Co^{2+} or vitamin B_{12} on the erythropoietin titer of mouse plasma is presented in Table 1. The result shows that Co^{2+} treatment produced about a three-fold increase in ^{59}Fe incorporation over the normal, whereas, vitamin B_{12} administration did not cause much change suggesting that vitamin B_{12} had no effect on the erythropoietin synthesis in normal animals. The effect of Co^{2+} on the ^{59}Fe incorporation may be due to increased erythropoietin synthesis or its direct effect on the marrow erythropoiesis as shown by Fisher [12] in isolated perfused hind legs of dogs. However ^{59}Fe incorporation is routinely used for the bioassay of erythropoietin [6—8, 13—19]. The result also shows that ^{59}Fe incorporation reached a steady level, 18 h after ^{59}Fe injection while the 4-h value was quite high, possibly due to the presence of non-incorporated ^{59}Fe which eventually got excreted within 18 h.

Since the above experiment indicates that the role of Co^{2+} is to elevate the erythropoietin level of plasma, attempts were made to isolate this erythropoietin from plasma as well as from kidney, which is the site of production of erythropoietin. The partially purified preparation from plasma or kidney had the ability to increase the ^{59}Fe incorporation significantly over the control value as shown in Table 2. Table 2 also shows that the kidney erythropoietin preparation stimulated ^{59}Fe incorporation by about 50% more than plasma erythropoietin. However, when the potency was calculated from the standard curve as shown in Fig. 1, the plasma erythropoietin was found to have a potency almost double that of the kidney erythropoietin (Table 2). This may be due to continuous production and release of erythropoietin from the kidney to the blood. The preparation obtained from normal plasma or kidney when tested for erythropoietin, revealed no further increase of ^{59}Fe incorporation over the saline-injected control value and this may be due to the fact that the starved rat assay is not sensitive enough to account for the normal concentration of erythropoietin present in blood or kidney.

The effect of this partially purified erythropoietin on the peroxidase activity is presented in Table 3, which shows that the peroxidase activity of both the

Table 1. *The effect of Co^{2+} or vitamin B_{12} on the erythropoietin level of plasma of normal mice*
2 μg Co^{2+} or 12 μg vitamin B_{12} per mouse was injected for six or three days respectively. The percentage ^{59}Fe incorporated was calculated assuming the blood volume of the mouse as 7% body weight. The results represent the mean value of two sets of experiments containing at least six animals in each group

Treatment	Amount of ^{59}Fe incorporated into red blood corpuscles after		
	4 h	18 h	24 h
	%	%	%
None	21	8	9
Co^{2+}	25	27	26
Vitamin B_{12}	28	12	10

Table 2. *The effect of partially purified erythropoietin on the ^{59}Fe incorporation into red blood corpuscles of starved rats*
Extraction of erythropoietin from normal as well as Co^{2+}-injected mouse plasma or kidney was carried out as mentioned under Materials and Methods. Potency was determined from the standard curve using sheep plasma erythropoietin as standard. The results of the ^{59}Fe incorporation represent the mean value of three sets of experiments containing three animals in each group. The percentage ^{59}Fe incorporated was calculated assuming the blood volume as 5% body weight of rat

Preparation	^{59}Fe incorporated	Control-corrected value	Protein injected	Potency
	%	%	mg	U/mg
None (saline)	16			
Normal plasma	16			
Cobalt-treated plasma[a]	31	15	0.6	1.6
Normal kidney	16			
Cobalt-treated kidney[a]	39	23	2.2	0.7

[a] The dose-response curves of these preparations were found to be parallel to the standard dose-response curve (Fig. 1).

Table 3. *Effect* in vivo *of partially purified erythropoietin on the peroxidase activity*
Erythropoietin was partially purified from plasma or kidney extract of normal or Co^{2+}-treated mice and injected into freash mice for this experiment. 0.4 U erythropoietin extracted from Co^{2+}-treated mice was injected for 6 days. Peroxidase activity was measured by the procedure as described earlier [1]. All values are mean ± S.E.

Source of erythropoietin	Peroxidase activity with			
	Guaiacol		*o*-Dianisidine	
	Thyroid	Submaxillary	Thyroid	Submaxillary
	$min^{-1} \times mg^{-1}$		$min^{-1} \times mg^{-1}$	
Plasma of normal animal	0.30 ± 0.03	0.15 ± 0.008	0.25 ± 0.02	0.27 ± 0.02
Plasma of Co^{2+}-treated animal	0.50 ± 0.07	0.21 ± 0.021	0.42 ± 0.05	0.55 ± 0.01
	$P < 0.02$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Kidney of normal animal	0.25 ± 0.02	0.12 ± 0.011	0.20 ± 0.01	0.25 ± 0.05
Kidney of Co^{2+}-treated animal	0.47 ± 0.08	0.21 ± 0.034	0.46 ± 0.08	0.56 ± 0.07
	$P < 0.01$	$P < 0.02$	$P < 0.05$	$P < 0.001$

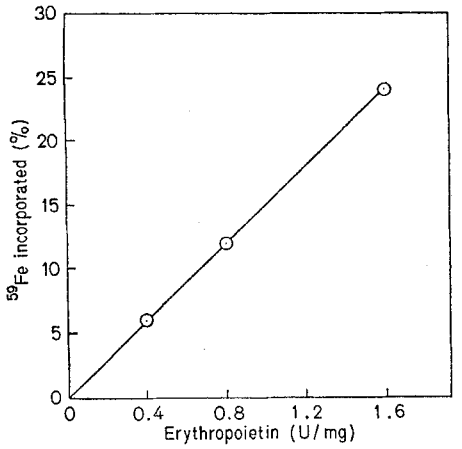

Fig. 1. *Standard curve of sheep plasma erythropoietin.* The assay of erythropoietin by ^{59}Fe incorporation study was described under Materials and Methods. Values given in the figure were corrected for the 16% ^{59}Fe incorporation value of normal animals. The data presented is the mean value of two sets of experiments containing at least three animals in each group

Table 4. *Effect* in vivo *of partially purified erythropoietin on the tyrosine iodinase activity*
Experimental conditions were same as mentioned in Table 3. The preparation and assay of the enzyme was reported earlier [1]. For the assay of tyrosine iodinase activity of the thyroid microsomal preparation, the incubation mixture contained in a final volume of 3 ml: 300 μmol phosphate buffer pH 7.4, 40 nmol KI, 20 μCi $Na^{131}I$, 4 μmol L-tyrosine, 2 μmol glucose, 250 μg glucose oxidase, 8 μg of enzyme preparation and water. In case of submaxillary gland, the incubation mixture contained the same except that 300 μmol phosphate buffer pH 6.5 and 250 μg soluble supernatant protein was used as the enzyme preparation. The tyrosine iodinase activity was measured as I^- incorporated in nmol $\times min^{-1} \times mg^{-1}$. All values are mean ± S.E.

Source of erythropoietin	Tyrosine iodinase activity	
	Thyroid	Submaxillary
	nmol $\times min^{-1} \times mg^{-1}$	
Plasma of normal animal	9.0 ± 0.5	16 ± 2.6
Plasma of Co^{2+}-treated animal	5.5 ± 0.3	24 ± 3.0
	$P < 0.02$	$P < 0.05$
Kidney of normal animal	9.5 ± 0.6	16 ± 1.5
Kidney of Co^{2+}-treated animal	6.8 ± 0.4	23.4 ± 2.8
	$P < 0.02$	$P < 0.02$

thyroid and submaxillary glands was significantly stimulated *in vivo* by this partially purified erythropoietin preparation. The tyrosine iodinase activity (Table 4) of the thyroid gland was inhibited to some extent while that of the submaxillary gland was significantly stimulated under the same condition.

The effect of partially purified erythropoietin from the Co^{2+}-treated mice on the above enzyme system tempted us to investigate whether preparations of erythropoietin from other sources could produce the same effect. Table 5 presents the effect *in vivo* of sheep plasma erythropoietin on the peroxidase activity of the thyroid and submaxillary glands. The result shows that erythropoietin significantly stimulated the peroxidase activity. Results with guaiacol and KI peroxidation suggest that the peroxidase, which we measured in these experiments, was a true peroxidase.

The activity of tyrosine iodinase was also investigated after erythropoietin administration. The result in Table 6 indicates that sheep erythropoietin *in vivo* inhibited the thyroid iodinase activity by 50% while it stimulated that of the submaxillary gland by 88% under identical condition.

The stimulation of peroxidase and tyrosine iodinase activity of the submaxillary gland by erythropoietin *in vivo* led us to investigate whether erythropoietin has any effect *in vitro* on these two enzymes activities. While studying its effect on peroxidase activity by *o*-dianisidine peroxidation, it was found

Table 5. *The effect* in vivo *of erythropoietin on the peroxidase activity*
0.4 unit of sheep erythropoietin was injected for 6 days. The preparation and assay of the enzyme activity were described under Materials and Methods. The peroxidase activity was measured as the change in absorbance $\times$ $min^{-1} \times mg^{-1}$. All values are mean $\pm$ S.E.

Treatment	Peroxidase activity					
	Guaiacol		*o*-Dianisidine		KI	
	Thyroid	Submaxillary	Thyroid	Submaxillary	Thyroid	Submaxillary
	$min^{-1} \times mg^{-1}$		$min^{-1} \times mg^{-1}$		$min^{-1} \times mg^{-1}$	
None	0.23 ± 0.01	0.14 ± 0.03	0.25 ± 0.10	0.17 ± 0.011	0.20 ± 0.02	0.47 ± 0.2
Erythropoietin	0.88 ± 0.05	0.46 ± 0.01	0.44 ± 0.03	0.46 ± 0.098	0.42 ± 0.07	1.60 ± 0.61
	$P < 0.02$	$P < 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.05$	$P < 0.01$

Table 6. *The effect* in vivo *of erythropoietin on tyrosine iodinase activity*
0.4 unit of sheep erythropoietin was injected for 6 days. The assay system was same as described in the legend of Table 4. The tyrosine iodinase activity was measured as the amount of I^- incorporated in nmol $\times$ $min^{-1} \times mg^{-1}$. All values are mean $\pm$ S.E.

Treatment	Tyrosine iodinase activity	
	Thyroid	Submaxillary
	nmol $\times$ $min^{-1} \times mg^{-1}$	
None	9.00 ± 0.50	16 ± 1.3
Erythropoietin	4.50 ± 0.40	30 ± 3.6
	$P < 0.05$	$P < 0.05$

Table 7. *Effect of erythropoietin* in vitro *on the peroxidase activity*
E represents enzyme preparation from submaxillary gland and Ep represents sheep erythropoietin. Assay of peroxidase activity was the same as described under Materials and Methods except that different concentration of erythropoietin was added before the addition of enzyme. The result is a typical one and verified in at least three experiments

Conc of sheep erythropoietin (in 3 ml)	Peroxidase activity					
	Guaiacol assay			I_3^- assay		
	E (1)	Ep (2)	E + Ep (3)	E (1)	Ep (2)	E + Ep (3)
unit	min^{-1}			min^{-1}		
0	0.046	—	0.046	0.10	—	0.10
0.04		0.002	0.056		0.002	0.14
0.08		0.004	0.077		0.005	0.15
0.16		0.010	0.090		0.005	0.16

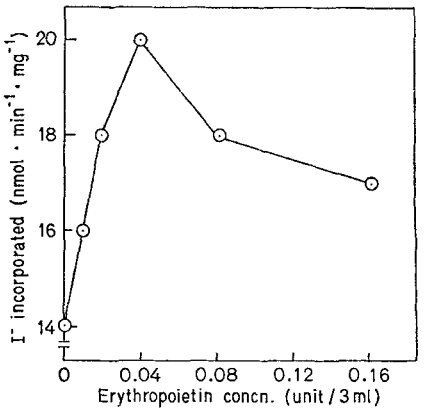

Fig. 2. *Effect of erythropoietin* in vitro *on the iodination of tyrosine by submaxillary enzyme.* The incubation mixture contained the same as described in the legend of Table 4. Erythropoietin of different concentration was added to the incubation mixture. The result is a typical one and verified in at least five experiments

that erythropoietin itself showed a strong pseudoperoxidase activity as evidenced by its significant nonenzymatic peroxidation of *o*-dianisidine. Hence peroxidase activity was finally measured using guaiacaol which is least sensitive to pseudoperoxidases and also using KI to measure the iodide peroxidase activity. Table 7 shows that erythropoietin still demonstrated a negligbile peroxidase activity towards guaiacol or KI peroxidation. However, at any particular concentration of erythropoietin, the peroxidase activity (column 3) was found to be greater than the additive peroxidase activities of either enyzme alone (column 1) or erythropoietin alone (column 2). Almost similar results were obtained in the case of I_3^- assay in the presence of erythropoietin. Fig. 2 demonstrates that the iodination reaction catalysed by the submaxillary supernatant enzyme preparation was also stimulated *in vitro* by sheep erythropoietin. The stimulation increased up to the concentration of 0.04 unit and thereafter the stimulation decreased. However, erythropoietin even up to a concentration of 0.16 unit did not increase the nonenzymatic iodination of tyrosine. Human urinary erythropoietin which is more purified and potent (2.6 U/mg) preparation, when added *in vitro,* also stimulated the iodination of tyrosine by the same enzyme preparation in a similar manner.

That this stimulatory effect on iodination reaction is due to erythropoietin and not due to other contaminants, is strengthened by the observation shown in Table 8. The result shows that when erythropoietin, being a glycoprotein, is treated with neuraminidase, dialysed overnight and added to the

Table 8. *Effect of neuraminidase on the stimulatory effect of erythropoietin on tyrosine iodination*
The assay system for the submaxillary enzyme was described in the legend of Table 4. In the experiment 3, 600 μg erythropoietin was incubated with 50 μg neuraminidase at 37 °C in 0.2 ml 0.05 M acetate buffer pH 5.0 for 30 min. The whole mixture was made up to 0.5 ml with distilled water and dialysed against 50 ml distilled water for 24 h to remove the liberated sialic acid. 0.083 ml of the dialysed material (equivalent to 100 μg erythropoietin) was added to the incubation mixture. The experiment 2 represents data obtained with erythropoietin in the above manner without neuraminidase. In the experiment 4 10 μg neuraminidase was added to the normal incubation mixture of experiment 1. The tyrosine iodinase activity was again measured as the amount of I^- incorporated in $nmol \times min^{-1} \times mg^{-1}$

System	Tyrosine iodinase activity
	$nmol \times min^{-1} \times mg^{-1}$
1. Enzyme	16.0
2. Enzyme + erythropoietin	22.6
3. Enzyme + erythropoietin (neuraminidase-treated)	15.8
4. Enzyme + neuraminidase	16.0

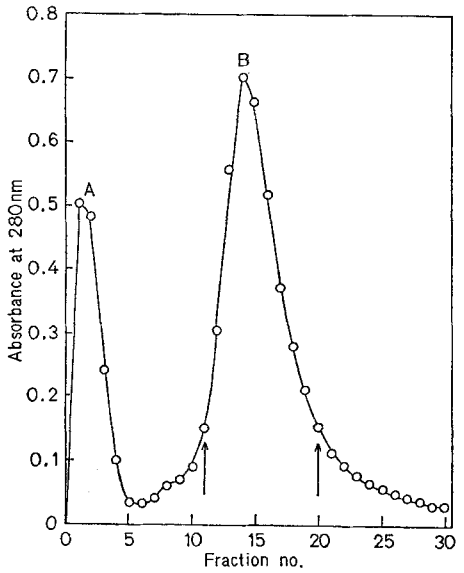

Fig. 3. *Elution profile of erythropoietin from DEAE-cellulose column.* The arrow indicates the fractions pooled and lyophilised

incubation system, the stimulatory effect of the erythropoietin was found to be abolished. Further, neuraminidase at this concentration has no effect on the enzymatic iodination of tyrosine. It was also observed that a purer preparation of erythropoietin, purified as mentioned below, required a lesser quantity to produce the same stimulation in comparison to that obtained with a relatively impure preparation. Step I erythropoietin (0.4 U/mg), when applied on DEAE-cellulose and eluted as described under Methods, resolved into two peaks, A and B (Fig. 3).

Table 9. *Effect of erythropoietin of two different specific activities on the iodination of tyrosine*
The assay of the submaxillary enzyme was same as described in the legend of Table 4. The activity of tyrosine iodinase was measured as the I^- incorporated in $nmol \times min^{-1} \times mg^{-1}$

System	Tyrosine iodinase activity
	$nmol \times min^{-1} \times mg^{-1}$
Enzyme	16
Enzyme + erythropoietin 100 μg [a]	23
Enzyme + erythropoietin 50 μg [b]	23

[a] 0.04 unit of step-I sheep erythropoietin (0.4 U/mg) was added to the incubation mixture.
[b] 0.04 unit of purified erythropoietin (0.8 U/mg) was added to the incubation mixture.

The lyophilized material corresponding to peak B was found to be erythropoietically more potent (0.8 U/mg). The purified erythropoietin showed a lesser number of contaminating protein bands, in comparison to the original step I material on polyacrylamide-gel electrophoresis (unpublished data). Using this purified erythropoietin at a concentration of half the original material, the percentage stimulation of iodination, was found to be the same as shown in Table 9.

DISCUSSION

In order to evaluate the possible mechanism of action of Co^{2+} or vitamin B_{12} on the peroxidase and tyrosine iodinase activity of the thyroid and submaxillary glands, several possibilities were considered: (a) the effect of Co^{2+} or vitamin B_{12} *in vivo* may be mediated at least partially through cobamide coenzyme, (b) Co^{2+} may be incorporated into the active enzyme molecule to alter its activity during its synthesis and (c) Co^{2+} or vitamin B_{12} *in vivo* may be responsible for the elaboration of a humoral factor in plasma which may have a secondary effect on the iodinating enzyme system. The role of cobamide coenzyme has already been reported earlier [1], but it is not the factor responsible for the cobalt effect presented in this communication. This results indicate that the effect is due to cobalt only. Regarding the second possibility, it has been observed that during preliminary purification of the peroxidase of the submaxillary gland after ^{60}Co administration and consequent isolation of its prosthetic part by *n*-butanone according to the method of Teale [20], Co^{2+} is not incorporated into the apoenzyme or prosthetic part of the enzyme during its synthesis *de novo*. The third possibility for a secondary humoral mechanism was therefore looked for.

Results, presented in Table 1, confirm the earlier observation that Co^{2+} has the ability to elicit a high plasma titer of erythropoietin [4]. Vitamin B_{12}, however, had no effect under identical conditions suggesting that vitamin B_{12} has no role in erythropoietin production.

Erythropoietin has previously been extracted from the plasma, urine and kidney of a variety of species under different conditions [7, 8, 13—19, 21]. We have also been able to extract the erythropoietin from plasma or kidney of Co^{2+}-treated mice, as evidenced by its ability to increase ^{59}Fe incorporation into red blood corpuscles (Table 2). Gordon *et al.* [22], however, believe that kidney contains a renal erythropoietic factor which is different from erythropoietin and this factor requires preincubation with plasma globulin to produce active hormone [23]. However, the material obtained from the kidney of Co^{2+}-treated mice, did not require any preincubation with plasma protein but produced significant ^{59}Fe incorporation when injected directly into the starved rat.

These partially purified erythropoietin preparations from cobalt-treated animals, when administered, gave rise to the response (Table 3 and 4) almost identical to our previous observation of Co^{2+} administration [1]. The stimulation of the peroxidase activity by these partially purified erythropoietin preparations indicates that the effect of Co^{2+} *in vivo* was mediated, to a large extent, through erythropoietin and not due to a cobalamine derivative. To support this hypothesis, the effect of sheep erythropoietin was noted on these enzyme systems, and the observed effect was the same as that of mouse erythropoietin (Table 5 and 6).

These results *in vivo* were then followed by some experiments *in vitro* where the sheep erythropoietin was found to stimulate the tyrosine iodination significantly using submaxillary gland homogenate as the enzyme preparation. Similar stimulation was also obtained using soluble supernatant as the enzyme preparation (Fig. 2). Human urinary erythropoietin also had the same effect. This result led us to investigate whether the peroxidase activity *in vitro* was affected similarly by sheep erythropoietin preparation. It was interesting to note that both the guaiacol peroxidation and I_3^- formation (Table 7) were stimulated in the presence of the erythropoietin.

Erythropoietin is a glycoprotein containing sialic acid on which the biological activity depends and removal of sialic acid by neuraminidase absolishes the potency of the material [24]. Erythropoietin lost its stimulatory effect on iodination when preincubated with neuraminidase (Table 8). This suggests that the effect is due to erythropoietin and not due to any contamination present in the erythropoietin preparation. This conclusion is further strengthened from the data presented in Table 9, where 50 μg of a partially purified preparation (0.8 U/mg) was required to produce the same stimulation as obtained with 100 μg of relatively impure material (0.4 U/mg).

The role of erythropoietin on erythrocyte differentiation along with hemoglobin biosynthesis is well known [5, 25—31]. However, its effect on peroxidase or tyrosine iodinase activity has not yet been reported. Peroxidases are usually hemoproteins containing heme as a prosthetic part [32—35]. It is therefore, very tempting to speculate that the effect of erythropoietin *in vivo* on the peroxidase is mediated through increased heme synthesis. As far as we know, every well-characterised peroxidase that has been tested shows both iodide peroxidase and tyrosine iodinase activity [32—34, 36]. The stimulation of peroxidase activity of mouse submaxillary gland by Co^{2+} through erythropoietin therefore reflects almost similar effects on its tyrosine iodinase activity. The thyroid peroxidase in the soluble supernatant fraction appears to be a non-specific peroxidase without having any catalysing effect on the iodination reaction. The same preparation did not catalyse the iodination even after an 18-h dialysis and also using H_2O_2 or glucose-glucose oxidase as H_2O_2 generator. The microsomal tyrosine iodinase may be different from this peroxidase and its inhibition by Co^{2+} *in vivo* may be due to a direct interaction of Co^{2+} with the enzyme molecule [1]. However, we are now unable to explain why erythropoietin has opposite effects on the tyrosine iosinase activity of thyroid and submaxillary gland.

It was known for a long time that cobalt salts alter thyroid function but until now, the mechanism has not been fully elucidated. From the present investigation, it may be suggested that the hypofunction of thyroid produced by Co^{2+} may be due not only to the direct action of Co^{2+} but also indirectly through the effect of erythropoietin on the thyroid iodinating enzyme.

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