of brain tissue. They suggest that separated portions of cerebral cortex from experimental animals or from man may be used in a wide range of investigations in which metabolic and electrical responses to stimuli are open to simultaneous investigation.

SUMMARY

1. Methods have been devised, using modified manometric apparatus, which enable electric stimuli to be applied to thin slices of brain tissue under good metabolic conditions.

2. The application for some hours of alternating currents of up to 3 V., or of brief condenser discharges of up to 30 V. several time per sec., between electrodes 2–10 mm. apart caused in the apparatus no changes which might be mistaken for metabolic responses.

3. When current was applied to electrodes in contact with brain-cortex slices, the respiration of the slices increased. Values up to double those of the initial respiration were observed.

4. The increased respiration could be maintained for some hours, and in a given experimental arrangement increased with increase in the voltage applied, when this was above a threshold value.

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Amino-acids and Proteins in Haemoglobin Formation

2. ISOLEUCINE

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Among essential amino-acids, the role of isoleucine in haematopoiesis is specially interesting, because, despite its absence in ox, human and horse globin (Brand & Grantham, 1946), this amino-acid has been shown to be essential for haemoglobin formation in weanling rats by Orten and collaborators (Orten, Orten & Bourque, 1945; Orten, Bourque & Orten, 1945). The present paper describes studies on the influence of isoleucine on the regeneration of haemoglobin and red cells in adult rats suffering from experimental anaemia, graded doses of isoleucine being administered to rats maintained on a diet containing horse globin as the sole source of protein and rendered anaemic by administration of

Table 1. *Recovery of rats from phenylhydrazine anaemia on a basal isoleucine-free diet and on diets containing different amounts of isoleucine*

(Red cell values in millions/cu.mm.; haemoglobin values in g./100 ml. blood; weight in g. Group means with their standard error.)

Exp. no.	No. of animals used	Isoleucine dosage (mg./rat/day)		Time after injection (days)									
				0	4	8	12	14	16	18	21	26	33
1	16	0	Red cell no.	6·37±0·19	3·66±0·26	4·41±0·58	5·32±0·48	—	5·93±0·79	6·19±0·38	6·41±0·44	5·72±0·54	4·66±0·79
			Haemoglobin	14·6±0·07	9·5±0·10	10·9±0·11	12·3±0·09	—	12·9±0·09	13·5±0·10	14·3±0·06	13·5±0·07	11·2±0·06
			Weight	134 ±7·8	121 ±7·5	113 ±7·1	—	107±6·5	—	—	101 ±6·2	91±5·2	83±5·1
2	8	10	Red cell no.	6·35±0·03	3·61±0·11	4·54±0·11	5·58±0·09	—	6·00±0·08	6·25±0·09	6·36±0·08	6·28±0·06	6·16±0·07
			Haemoglobin	14·6±0·12	9·5±0·19	11·1±0·23	12·6±0·16	—	13·2±0·16	14·4±0·13	14·6±0·09	14·5±0·12	14·1±0·10
			Weight	130 ±12·2	118 ±10·9	112 ±10·7	—	106±10·2	—	—	102±10·0	—	—
3	8	20	Red cell no.	6·39±0·04	3·47±0·09	4·38±0·06	6·37±0·05	—	6·43±0·05	6·44±0·05	—	—	—
			Haemoglobin	14·6±0·06	9·4±0·08	12·4±0·06	14·6±0·07	—	14·8±0·08	14·8±0·08	—	—	—
			Weight	136 ±7·3	125 ±6·8	119 ±6·9	—	115±5·7	111 ±6·1	—	—	—	—
4	10	20 (by injection)	Red cell no.	6·44±0·08	3·75±0·08	4·54±0·09	6·45±0·08	—	6·74±0·08	—	—	—	—
			Haemoglobin	14·2±0·08	9·8±0·16	11·7±0·19	14·1±0·07	—	14·4±0·06	—	—	—	—
			Weight	126 ±11·1	113 ±9·4	106 ±9·0	—	101±8·8	98±8·7	—	—	—	—
5	8	40	Red cell no.	6·43±0·04	3·42±0·05	4·38±0·05	6·43±0·04	—	6·47±0·04	6·50±0·03	—	—	—
			Haemoglobin	14·7±0·17	9·3±0·10	12·4±0·19	14·6±0·16	—	14·8±0·13	14·8±0·11	—	—	—
			Weight	139 ±9·2	126 ±8·7	122 ±8·4	—	119 ±8·1	116 ±7·8	—	—	—	—
6	8	100	Red cell no.	6·63±0·08	3·67±0·06	4·56±0·07	6·55±0·09	—	6·68±0·12	6·65±0·13	—	—	—
			Haemoglobin	14·4±0·08	9·4±0·11	11·9±0·15	14·4±0·07	—	14·5±0·09	14·7±0·07	—	—	—
			Weight	130 ±11·1	116 ±10·1	126 ±10·8	—	132 ±10·8	134 ±10·9	—	—	—	—

phenylhydrazine according to the technique described (Yeshoda & Damodaran, 1947). The effect of prolonged isoleucine deficiency on haematopoiesis in normal adult animals was also studied.

EXPERIMENTAL

Preparation of horse globin. Globin was prepared by a combination of the methods of Schulz (1898) and Anson & Mirsky (1930); it was found that when the latter method was used exclusively, very large volumes of acetone were required to give a satisfactory product. Horse red corpuscles, freed from serum by centrifuging of defibrinated blood and repeated washing with 0·9% (w/v) NaCl, were laked by the addition of ether and, after centrifuging to remove the stroma, dialysed free from NaCl. The solution was now treated at 0° with ice-cold 0·1 N-HCl in quantity just sufficient to redissolve the flocculent precipitate formed, the amount of acid required being determined by preliminary titration of a measured fraction. Half the volume of ethanol was added and then the pH brought carefully to 7·5 by the

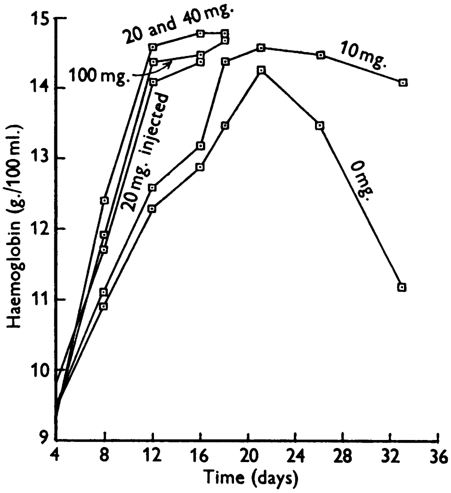

Fig. 1. Regeneration of haemoglobin after phenylhydrazine administration at varying daily levels of isoleucine. All the figures are mean values (for details see Table 1).

dropwise addition of NH_3. The precipitate formed was separated by decantation and centrifuging, washed free from haemin with acidified acetone (1 vol. of HCl/100 vol. of acetone), filtered at the pump and dried by washing with acetone and ether. The globin so prepared contained 0·4% ash and 16·85% N (moisture-free).

Preparation of isoleucine. L-Isoleucine was first prepared from casein phosphopeptone available from previous work (Damodaran & Ramachandran, 1941) by the method then described. Later it was prepared directly from casein. Casein was hydrolysed in the usual way with H_2SO_4, and after removal of acid with $Ba(OH)_2$, the solution was evaporated till tyrosine separated out. The filtrate was further concentrated to give a crystalline mixture of leucines. Isoleucine was isolated via the methanol-soluble Cu salt (Damodaran, 1931).

Experimental diets and animals. Experiments were carried out on rats from the stock colony weighing from 100 to 175 g., animals being as far as possible evenly distributed in groups regarding age, weight and sex. The isoleucine-free basal diet was made up as follows: horse globin, 18; tapioca starch, 40; sugar, 20; melted butter fat, 17; salt mixture, 5%.

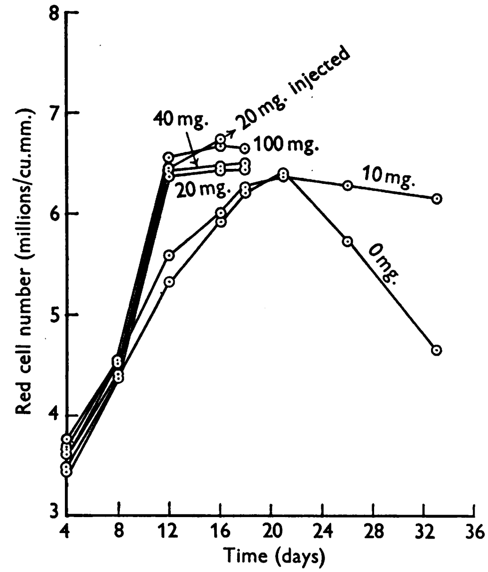

Fig. 2. Regeneration of erythrocytes after phenylhydrazine administration at varying daily levels of isoleucine. All the figures are mean values (for details see Table 1).

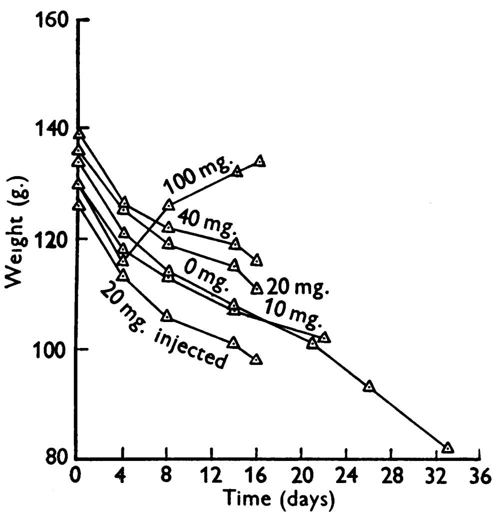

Fig. 3. Body weights of rats recovering from phenylhydrazine treatment at different daily levels of isoleucine. All the figures are mean values (for details see Table 1).

The latter was the Steenbock-Nelson salt mixture no. 40 + 0·03% $CuSO_4, 5H_2O$ (Steenbock & Nelson, 1923; Pearson, Elvehjem & Hart, 1937). Each rat received in addition

Table 2. *Effect of prolonged isoleucine deficiency in normal animals*

(Red cell values in millions/cu.mm.; haemoglobin in g./100 ml. blood; body weight in g.)

		Time (days)								
Rat no.		0	15	30	40*	45	50	55†	70	80
59	Red cell no.	7·37	6·27	4·71	4·02	5·22	6·41	7·08	5·41	4·08
	Haemoglobin	15·3	13·5	10·3	9·3	10·8	12·2	14·5	13·2	9·9
	Weight	184	149	130	114	—	110	94	71	53
60	Red cell no.	7·16	6·05	4·58	3·74	4·88	6·38	6·99	5·47	4·21
	Haemoglobin	15·2	13·5	10·4	9·3	10·6	12·1	14·6	13·3	10·1
	Weight	176	136	119	100	—	88	83	68	50
61	Red cell no.	6·58	5·37	4·42	3·81	4·83	6·44	7·11	Died	—
	Haemoglobin	14·6	13·2	10·1	8·9	10·6	13·1	15·2		
	Weight	109	72	68	59	—	51	48		
62	Red cell no.	6·66	5·41	3·63	3·14	4·46	6·57	6·97	5·53	Died
	Haemoglobin	14·4	13·4	10·5	9·5	10·4	12·8	14·7	13·1	
	Weight	128	93	70	61	—	57	52	41	
63	Red cell no.	6·82	5·58	4·11	3·35	4·67	6·84	7·14	5·68	Died
	Haemoglobin	14·8	13·2	10·2	9·1	10·5	12·8	14·6	13·7	
	Weight	110	81	70	53	—	48	42	38	
64	Red cell no.	6·30	5·17	3·42	Died	—	—	—	—	—
	Haemoglobin	14·3	13·0	9·8						
	Weight	104	88	63						
65	Red cell no.	6·28	5·04	3·39	Died	—	—	—	—	—
	Haemoglobin	14·4	13·1	8·6						
	Weight	104	68	51						
66	Red cell no.	6·49	5·39	4·61	3·87	5·17	6·57	6·81	5·58	Died
	Haemoglobin	14·5	13·3	10·4	9·5	10·7	12·9	14·2	13·1	
	Weight	106	88	69	51	—	46	42	39	
67	Red cell no.	7·08	5·83	4·17	3·44	4·82	6·48	6·96	5·51	Died
	Haemoglobin	14·8	13·2	10·3	9·3	10·6	12·7	14·5	13·3	
	Weight	138	100	81	64	—	48	44	39	
68	Red cell no.	6·71	5·64	4·08	3·51	4·78	6·29	6·92	5·62	Died
	Haemoglobin	14·6	13·2	9·9	9·1	10·6	12·8	14·4	13·1	
	Weight	117	81	52	64	—	58	53	44	
69	Red cell no.	6·56	5·24	3·89	Died	—	—	—	—	—
	Haemoglobin	14·2	13·4	9·9						
	Weight	104	65	52						
70	Red cell no.	6·37	5·21	3·76	Died	—	—	—	—	—
	Haemoglobin	14·3	13·2	9·3						
	Weight	103	60	43						

* Isoleucine supplemented. † Isoleucine withheld.

a standard cod liver oil concentrate corresponding to 180 i.u. of vitamin A and 30 i.u. of vitamin D/day, and the following supplements of vitamins of the B complex/day: 25 μg. aneurin hydrochloride, 20 μg. riboflavin, 20 μg. pyridoxin, 100 μg. calcium pantothenate and 100 μg. nicotinic acid. The animals on the isoleucine diet received daily in addition to the above, isoleucine at different levels administered separately. The food was provided *ad lib.* and the daily intakes recorded. The animals were weighed twice a week. The techniques used for the production of anaemia and estimation of red cells and haemoglobin were as described previously (Yeshoda & Damodaran, 1947).

Experiments on anaemic animals. Six groups of animals were used. The control group consisting of sixteen animals was maintained on the isoleucine-free basal diet. Four groups, each of which contained eight animals, received in addition to the basal diet 10, 20, 40 and 100 mg., respectively, of L-isoleucine/rat/day administered orally. To the sixth group 20 mg. of the amino-acid were given by subcutaneous injection. Initial red cell and haemoglobin values were determined in blood from the tail vein on 3 consecutive days preceding experiment. The animals were placed on the basal diet on the day on which phenylhydrazine was injected; isoleucine supplements were given from the 4th day when the peak of anaemia was reached. Determination of erythrocytes and haemoglobin were made every 4th day till the animals recovered from anaemia or till it became evident that there was no possibility of recovery. The mean values for haemoglobin, red cells and body weights for the six groups of animals are given in Table 1 and represented graphically in Figs. 1, 2 and 3.

Experiments on normal animals. A group of twelve animals was kept on the isoleucine-free basal diet and observations on red cells, haemoglobin and weights made for a period of 70–80 days. The results are recorded in Table 2 and Fig. 4.

RESULTS AND DISCUSSION

It will be seen from Table 1 and Figs. 1 and 2 that the course of recovery of animals from anaemia is clearly dependent upon the presence as well as upon the quantity of isoleucine in the diet. The animals on the isoleucine-deficient diet recovered their normal haemoglobin and red cell values on the 21st day of the experiment, or 17 days after the most acute stage of anaemia. The same was true of the animals receiving 10 mg. of isoleucine per day. On 20 mg. or more of isoleucine per day complete recovery took place within 8 days. Injected isoleucine also brought about recovery in the same length of time.

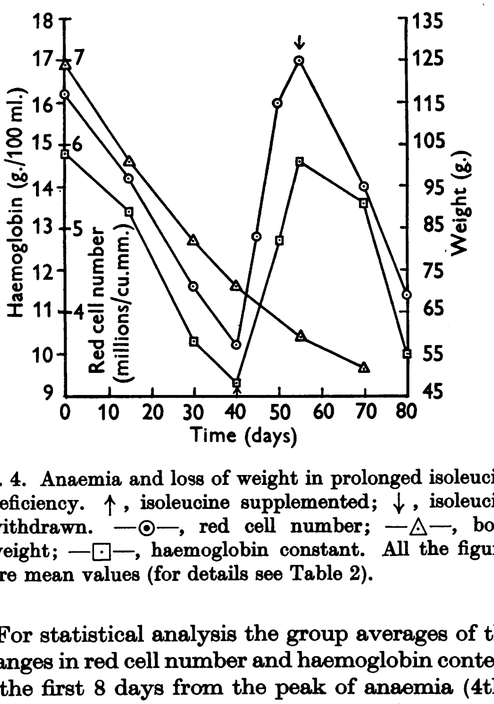

Fig. 4. Anaemia and loss of weight in prolonged isoleucine deficiency. ↑, isoleucine supplemented; ↓, isoleucine withdrawn. —⊙—, red cell number; —△—, body weight; —⊡—, haemoglobin constant. All the figures are mean values (for details see Table 2).

For statistical analysis the group averages of the changes in red cell number and haemoglobin content in the first 8 days from the peak of anaemia (4th–12th day), calculated as percentages of the initial values, given in Table 3 were used. 'Student's' test was applied for the calculation of 't'. Similar calculations were made for the percentage changes in weights also, but for a period of 10 days from the peak of the anaemia.

The values of 't' given in Table 4 leave no doubt as to the reliability of the interferences stated above. The analysis shows in addition that there is no significant difference in the rate of regeneration of erythrocytes or of haemoglobin at isoleucine intakes above 20 mg. As between injection and oral administration statistical analysis shows that the latter is significantly more effective for haemoglobin formation, although the overall time of recovery was about the same in both the cases.

Table 3. *Change in haemoglobin, red cells and body weight expressed as percentages of initial values*

Exp. no.	Dosage used (mg.)	Red cells 4–12th day	Haemoglobin 4–12th day	Weight 4–14th day
1	0	26·1	18·9	−11·8
2	10	32·4	21·3	− 9·2
3	20	45·3	37·1	− 7·5
4	20 (injected)	41·8	30·6	−10·2
5	40	46·7	36·4	− 5·1
6	100	43·3	35·2	+12·4

The amount of isoleucine administered has a pronounced effect on the weights of the animals, in marked contrast to the results previously reported on tryptophan. With the latter it was found that 20 mg. of the amino-acid per day, which was required for normal haematopoiesis, was also sufficient to prevent loss of weight of the animals recovering from phenylhydrazine anaemia, except when the tryptophan was given by injection. A difference of the same kind between oral and parenteral administration was observed in the case of isoleucine too, but even under conditions of oral administration loss of weight was prevented only at a level of 100 mg. of isoleucine, although a fifth of this quantity was found sufficient for normal haematopoiesis. These observations are in accord with the results of Frost & Sandy (1948) who, in assays on the optimum level of tryptophan and isoleucine in partial hydrolysates of fibrin, found the requirement of tryptophan for maximum gain in weight to be 18–20 mg. per day and of isoleucine somewhat greater than 75 mg.

In another important respect also the effects observed in isoleucine deficiency differ from those

Table 4. *Statistical analysis of percentage changes in haemoglobin, red cells and body weight*

(Values of 't'.)

		Haemoglobin					Red cells					Weight				
Isoleucine dosage ...		10	20	20 (injected)	40	100	100	40	20 (injected)	20	10	100	40	20 (injected)	20	10
Dosage compared with	0	1·66	10·43	8·17	12·73	10·58	14·45	23·51	12·14	16·13	3·58	14·65	4·27	1·08	2·71	1·61
	10	—	9·03	7·27	19·16	10·62	4·16	6·12	3·69	4·93	—	21·28	6·18	1·09	2·31	—
	20	—	—	3·63	0·43	0·98	1·09	0·99	1·81	—	—	21·92	4·99	3·30	—	—
	40	—	—	—	—	1·06	2·43	—	—	—	—	20·80	—	—	—	—

reported with regard to tryptophan. Although the recovery of animals from anaemia was greatly retarded by the absence of tryptophan, the blood picture was ultimately restored to normal, presumably at the expense of body substance as the animals showed great loss in weight. In agreement with this result attempts to make normal animals anaemic by removal of tryptophan from the diet were unsuccessful. Rats maintained on a tryptophan-deficient diet lost weight and died in 58–75 days, but throughout the experimental period exhibited no anaemia, the haemoglobin content and erythrocyte count showing no statistically significant difference from animals on a complete diet. In isoleucine deficiency, on the other hand, the recovery from phenylhydrazine anaemia was not only delayed, but was also temporary (Table 1 and Figs. 1 and 2). The blood picture was restored to normal on the 21st day of the experiment, but marked anaemia set in about the 33rd day. These results were fully confirmed by experiments on normal animals maintained on globin for an extended period. The results of this experiment which are detailed in Table 2 and Fig. 4 show that the blood picture rapidly responds to the presence and absence of isoleucine. Noticeable fall in haemoglobin and red cell values took place in a fortnight on an isoleucine-deficient diet, and in the course of about 40 days acute anaemia comparable to that produced with phenylhydrazine set in. On addition of 20 mg. of the amino-acid the blood picture returned to normal in 15 days. By the withdrawal of isoleucine from the diet anaemia could be again induced in these animals which died at the end of 70–80 days in the second period of isoleucine deficiency.

SUMMARY

1. The effect of administering isoleucine at various levels was studied on growth, red cell number and haemoglobin content of animals made anaemic by phenylhydrazine injection and maintained on a basal diet containing no isoleucine. The effect of prolonged isoleucine deficiency on normal animals was also investigated.

2. It was found that isoleucine is essential for haematopoiesis in rats, regeneration of erythrocytes and haemoglobin in animals suffering from experimental anaemia being greatly retarded in the absence of this amino-acid.

3. The quantity of isoleucine required for maintenance of weight was much greater than that required for haematopoiesis. Administration of quantities of isoleucine greater than 20 mg. per rat per day did not further accelerate red cell and haemoglobin formation, whilst for prevention of loss in weight about 100 mg. was found necessary.

4. Injected isoleucine was utilized for haemopoiesis, but was less effective than isoleucine administered by mouth.

5. Normal animals became markedly anaemic when maintained on an isoleucine-deficient diet for more than a few weeks.

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