Further Studies on Lysine Deficiency in Rats

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In an earlier paper (Harris, Neuberger & Sanger, 1943) the effects in young rats of a diet containing only small amounts of lysine were studied. The main symptoms of this deficiency were: cessation of growth without any marked loss of weight, wasting of muscular tissue, retarded bone formation, and reduced mitotic figures in the testes. Serum protein values were well below normal and there was also a reduction in the number of red cells and the amount of haemoglobin per unit volume of blood. The interpretation of the haematological data was rather difficult, because both haemoglobin and the number of red cells increase considerably from birth to pubescence, when the adult values are reached (Metcoff & Favour, 1944; Albanese, Holt, Kajdi & Frankston, 1943). A similar increase is also found in the protein content of plasma and it was therefore possible that the low values found in these lysine-deficient rats might have been due to a retention of infantile characteristics caused by the cessation of growth and were not indicative of a true anaemia or hypoproteinaemia. The experiments to be reported in this paper were, therefore, undertaken with older animals in which the number of red cells and the content both of haemoglobin and plasma proteins approached constancy. The absence of lysine from the diet was also more rigorously secured than in the earlier experiments.

The nutritional requirements for a particular amino-acid are usually measured by recording the changes of body weight of an experimental animal on diets in which the intake of that amino-acid is varied. It is obvious that changes of body weight will give only a very crude idea about variations of the protein content of cells and of that of the body as a whole. Thus it appeared possible that, since a diet deficient in an essential amino-acid produces a hypoproteinaemia, this might be associated with an oedema of the tissues. The increased water content of the body would then largely mask a real loss of weight, i.e. a loss of body substance. Moreover, as pointed out by Mitchell & Carman (1926), a relative increase of fat produced by a diet will also either mask or exaggerate a net gain or loss of body protein, if body weight is used as a criterion. It seemed, therefore, important to estimate both the water and the nitrogen content of the tissues; such measurements appeared to be particularly important in the

case of the liver, which occupies such a central position in protein metabolism.

In the earlier paper the question was discussed how far the observed changes in lysine deficiency were due directly to a lack of this amino-acid in the diet and how far they were primarily the result of a decreased food intake brought about by the amino-acid deficiency. We have therefore compared, in the present work, one group of rats on a lysine-deficient diet given food *ad libitum* with a second group receiving the same diet supplemented with lysine, the food also being given *ad libitum*; a third group of animals received the stock laboratory diet which was so restricted in amount as to produce a decrease in weight similar to that observed in the experimental group. This represents a modification of the paired-feeding method, a modification in which the body weights and not the food consumptions are equalized. Any differences found between the first and third groups could be considered to be caused specifically by the lack of lysine, and not by a restriction of food intake.

EXPERIMENTAL

Three litters of albino rats of the Wistar strain, with weights varying between 110 and 140 g., were obtained from Glaxo Laboratories. The animals were distributed uniformly with respect to sex among the different groups. They were kept in screened cages to exclude coprophagy. Weights were recorded twice weekly, and food consumption was also measured.

Diet. The basal diet (ZT) was as follows: zein 18%, maize starch 60.8%, arachis oil 14.4%, cod-liver oil 1.6%, *l*(-)-tryptophan 0.2% and salt mixture (U.S. Pharmacopoeia II, no. 2) 5%. In the control diet (ZTL) 0.5% of the starch in the basal diet was replaced by the same amount of *l*-lysine hydrochloride which had been prepared in this laboratory and had the correct composition and optical rotation. The water-soluble vitamins were given as a separate supplement in the following daily doses: aneurin 60 µg., pyridoxin 60 µg., riboflavin 100 µg., calcium *d*-pantothenate 200 µg., inositol 2 mg., nicotinamide 2 mg., choline chloride 6 mg. No additional concentrates were given to provide folic acid or biotin. The stock diet (SR) which was given in restricted amounts to a third group of rats had the following composition: rolled or ground oats 30%, whole-meal flour 35%, dried yeast 6%, dried skim milk 15%, meat and bone meal 10%, cod-liver oil 2% and salt mixture 2%.

Haemoglobin and red cell number. These were estimated at the beginning and end of the experiment and also at infrequent intervals during the experimental period. Measurements during the life of the rats were reduced to a minimum in order to avoid the loss of protein resulting from the loss of blood. Free-running blood was obtained by incision of the tail. All haemoglobin estimations were carried out by the same observer, using all necessary precautions to produce good matching and reproducible readings. A standard checked by the National Physical Laboratory was used and it was assumed that a reading of 100% corresponded to a haemoglobin content of 14.8 g./100 ml. of blood (King, Gilchrist & Matheson, 1944).

Protein, fat and water content. The three litters were killed by inhalation of carbon monoxide after 63, 75 and 85 days respectively. Blood was removed from the heart and used for the estimation of plasma proteins by the method of Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald (1943). The application of this method to rat plasma may involve a systematic error, as pointed out by Metcalf & Favour (1944) which, however, is likely to be constant. Comparison with results obtained by the Kjeldahl method is difficult, since the conversion factor for rat plasma protein is not well established. The liver was taken out, rinsed under the tap, and the excess water then removed by wiping on a towel. The liver was then weighed, cut into small slices, reweighed and the dry weight then estimated after drying to constant weight in a vacuum oven at 100°. Fat was estimated by extracting the dry liver powder with ether in a Soxhlet apparatus for 8 hr. The term 'fat' used in this paper denotes material which is extracted by ether under these conditions. The defatted residue was then used for

the estimation of N by the Kjeldahl method. The 'protein' content was calculated by multiplying the N content by 6.25. It is realized that this factor is arbitrary and also that the true protein-N content is smaller than the total N. It was found, however, that the non-protein-N is small, about 6-8% of the total N, both in liver and carcass, and that the relative proportion of non-protein to protein-N is not significantly altered by the diets used.

Skin, tail, head and intestinal organs were then removed, and the remaining carcass then cut up and dried as described for the liver. Fat and protein contents were then estimated as described above.

RESULTS

Body weights. Table 1 shows that the rate of growth of rats receiving a zein diet supplemented with tryptophan and lysine (ZTL) varies considerably even in rats of the same litter. The average increase of weight, which amounts to about 8 g./week, is similar to the value found for younger animals (Harris *et al.* 1943) at the same protein level in the diet. The food consumption runs parallel to the rate of growth and it thus appears that the variation in growth rates of the different rats is caused by differences in appetite. The rats receiving a diet which does not contain lysine (ZT) lose weight at varying rates; the average weekly loss of weight as percentage of the initial weight is about 1.5%, which compares well with a value of 1.8% found for

Table 1. *Changes of weight, red cell number, haemoglobin and plasma protein contents of rats receiving zein diets with (ZTL) and without (ZT) added lysine and of rats on a stock diet with restricted food intake (SR)*

(The letters A, B and C in column 1 refer to the three litters used.)

Rat no.	Body weight (g.)		Days on diet	Diet	Blood haemoglobin (g./100 ml.)		Red cells (millions/cu.mm.)		Plasma protein (%)	Average daily food consumption (g.)
	Initial	Final			Initial	Final	Initial	Final		
A 1 ♀	106	170	75	ZTL	13.6	14.2	7.98	8.48	7.03	12.3
A 6 ♂	151	279	75	"	12.7	15.1	7.82	8.96	7.03	17.5
A 8 ♂	137	224	75	"	13.0	16.3	7.72	9.67	5.95	18.6
B 9 ♂	140	256	63	"	13.8	14.2	7.48	8.59	7.15	16.7
B 13 ♀	113	167	63	"	14.5	15.1	7.40	8.96	6.36	11.6
C 17 ♂	119	151	85	"	13.0	14.5	8.10	8.00	6.04	9.6
	Mean value				13.5	14.9	7.75	8.76	6.58	12.2
A 2 ♀	111	102	75	ZT	13.6	13.0	8.92	7.12	5.49	9.2
A 7 ♂	140	115	75	"	13.0	10.7	7.76	6.56	5.15	9.4
B 11 ♂	136	113	63	"	13.0	10.3	6.66	7.20	4.75	7.8
B 12 ♀	110	97	63	"	13.3	9.5	6.84	7.12	5.10	9.3
B 15 ♀	110	88	63	"	14.2	10.3	7.36	5.46	5.95	8.1
C 16 ♂	111	88	85	"	13.0	11.8	6.86	7.64	5.80	7.1
C 18 ♀	107	92	85	"	13.3	12.1	8.00	8.00	5.01	6.0
	Mean value				13.4	11.1	7.48	7.10	5.18	8.1
A 3 ♀	109	111	75	SR	13.0	16.0	9.24	9.92	6.96	10.3
A 4 ♂	124	103	75	"	13.0	14.5	7.72	8.63	5.90	10.3
A 5 ♂	139	103	75	"	13.3	16.0	6.56	9.84	5.70	10.3
B 10 ♂	130	115	63	"	12.7	12.1	7.52	8.60	5.82	10.3
B 14 ♀	130	117	63	"	13.0	15.7	7.48	9.44	6.15	10.3
C 19 ♀	101	91	85	"	13.6	15.7	7.82	8.76	6.31	10.3
C 20 ♀	113	113	85	"	12.7	15.4	6.90	7.60	5.76	10.3
	Mean value				13.1	15.1	7.60	8.96	6.08	10.3

heavier animals (Neuberger & Webster, 1945). The restriction of food consumption for the animals on the stock diet (SR) to a uniform level has not succeeded in producing identical losses of body weight; the average decrease of weight is slightly less than in the ZT group. Histological examination of the liver, kidneys, thyroid, bone-marrow and testis was kindly carried out by Dr L. E. Glynn. There were no consistent histological changes associated with the different dietary groups. The ZT group showed a tendency to testicular atrophy with reduced spermatogenesis; but these changes occurred only in some animals and similar changes, though to a lesser extent, occurred in some animals on the reduced stock diet.

Haemoglobin and red cells. In spite of the appreciable variations which occurred within one group it can be seen from Table 1 that the average initial figures both for haemoglobin and red cells are similar for the three groups. Both haemoglobin content and cell number rise during the experiment in groups ZTL and SR to the full normal adult values, though the rise of the two sets of figures is not parallel for individual rats. Restriction of food intake on an otherwise adequate diet does not therefore lead to anaemia, although it produces a loss of body weight. The lysine-deficient animals (group ZT), however, show a marked fall both in haemoglobin content and number of red cells, and the final average values for haemoglobin are about 30% below those of the two control groups, whilst the average difference for cell number is about 20%. The decrease in haemoglobin content is slightly greater than the drop in cell number. The ratio of haemoglobin content, in g./100 ml., and the number of red cells, in millions/cu.mm., can be used as a measure of the 'colour index', i.e. for the average haemoglobin content of the individual erythrocyte. This ratio tends to be lower in lysine-deficient rats, although individual variations are considerable. It may, therefore, be said that this anaemia is probably of the hypochromic type. Haemoglobin estimations carried out during the experimental period indicated that this anaemia does not develop until the animals have been on the lysine-deficient diet for at least 40 days. Similar observations have been made on the anaemia produced in rats by dietary deficiency of tryptophan (Albanese *et al.* 1943).

Plasma proteins. The plasma protein values also vary considerably in each group. In the ZT group the figures vary between 4.75 and 5.95% with a mean value of 5.18%; the variations in the two controls are 5.7-7.15% and 5.7-7.0%, with mean values of 6.4 and 6.1% respectively. If litter-mates are compared the differences are even more significant. There is therefore an average reduction of plasma proteins in lysine-deficient animals of about 15-20% as compared with the two control groups.

The hypoproteinaemia is less severe than that found in younger animals (Harris *et al.* 1943).

Water content of the carcass and the liver. The part of the carcass used for these estimations represents only about 50% of the total body weight; hair and skin have also been examined in a number of rats; but since no additional information of any interest has been obtained, these results are not reported here. The water contents of the carcasses for all three groups are about equal and within normal limits (Table 2). The water content of the livers of

Table 2. *Water, fat and protein contents of carcasses of rats receiving normal (ZTL), lysine-deficient (ZT) diets and stock diets with restricted food intake (SR)*

Rat no.	Diet	Carcass			
		Weight (g.)	Fat content (%)	Water content (g./100 g. fat-free carcass)	Protein content (g./100 g. fat-free carcass)
A 1 ♀	ZTL	77.8	18.0	72.1	21.1
A 6 ♂	"	126.3	19.5	72.5	20.5
A 8 ♂	"	98.5	16.0	72.5	21.1
B 9 ♂	"	117.1	17.5	73.2	20.9
B 13 ♀	"	78.4	12.5	73.9	19.7
Mean value			16.7	73.0	20.7
A 2 ♀	ZT	47.3	11.7	73.6	19.7
A 7 ♂	"	54.2	8.6	73.7	19.6
B 11 ♂	"	42.9	8.3	73.8	20.0
B 12 ♀	"	42.9	12.4	73.3	20.3
B 15 ♀	"	36.1	8.1	73.7	19.8
Mean value			9.8	73.6	19.9
A 3 ♀	SR	53.7	8.4	72.6	20.3
A 4 ♂	"	46.6	1.3	71.5	20.1
A 5 ♂	"	46.2	1.3	72.8	19.4
B 10 ♂	"	53.5	2.9	73.1	20.0
B 14 ♀	"	52.5	7.2	73.0	20.0
Mean value			4.3	72.6	20.0

the lysine-deficient animals (ZT) tends to be higher than in the two control groups (Table 3). In view of the variations found, it is, however, doubtful whether the difference is of statistical significance. If there is any increased water retention in the livers of ZT animals as compared with the controls, it is only very small.

Fat content of the carcass and the liver. The fat content both of the carcass and of the liver differs considerably for the different groups. The animals on the restricted stock diet have very low fat contents; the fat deposits have practically disappeared. The fat content of the livers of the rats of the ZTL is fairly high, particularly if compared with that of the lysine-deficient group (ZT). The high fat content found in some of the livers of the ZTL group was rather unexpected and the design of the experiment does not permit us to establish with certainty the reason for the occurrence of these fatty livers. All

Table 3. *Water, fat and protein content of livers of rats receiving normal (ZTL), lysine-deficient (ZT) diets and of rats on stock diet with restricted food intake (SR)*

Rat no.	Diet	Liver					
		Weight (g.)	Fat content (%)	Fat-free tissue (g./100 g. body wt.)	Water content (g./100 g. fat-free tissue)	Protein content	
						g./100 g. fat-free tissue	g./100 g. body wt.
A 1 ♀	ZTL	5.331	10.2	2.81	73.1	20.3	0.573
A 6 ♂	"	9.608	6.9	3.02	75.0	20.3	0.615
A 8 ♂	"	8.781	3.5	3.41	76.3	19.1	0.672
B 9 ♂	"	9.709	16.4	3.06	73.7	21.2	0.650
B 13 ♀	"	7.076	17.5	3.50	75.2	19.2	0.672
C 17 ♂	"	4.854	5.5	3.04	69.9	19.8	0.601
Mean value			10.0	3.14	73.9	20.2	0.631
A 2 ♀	ZT	3.372	6.1	3.11	74.3	16.9	0.527
A 7 ♂	"	3.798	5.0	3.15	75.0	15.9	0.501
B 11 ♂	"	4.338	5.7	3.62	75.1	15.8	0.571
B 12 ♀	"	3.419	4.0	3.38	74.0	17.6	0.584
B 15 ♀	"	2.765	4.2	3.01	75.9	17.9	0.530
C 16 ♂	"	2.876	3.8	3.15	74.7	16.1	0.506
C 18 ♀	"	2.421	5.2	2.50	73.3	20.1	0.503
Mean value			4.9	3.28	74.8	17.2	0.533
A 3 ♀	SR	3.172	3.2	2.79	72.3	21.3	0.596
A 4 ♂	"	3.040	3.3	2.86	73.9	20.7	0.593
A 5 ♂	"	2.908	2.1	2.77	72.7	21.1	0.585
B 10 ♂	"	3.462	1.0	2.97	73.0	21.0	0.623
B 14 ♀	"	3.125	2.3	2.61	73.3	23.1	0.613
C 19 ♀	"	2.778	1.8	3.01	70.7	20.4	0.618
C 20 ♀	"	3.184	2.5	2.76	13.1	23.2	0.640
Mean value			2.3	2.82	72.7	21.5	0.610

the rats received choline, though possibly a sub-optimal amount. Zein is known to have only a weak lipotropic effect (Channon, Loach, Loizides, Manifold & Soliman, 1938) and this, together with the low choline and rather high aneurin content of the diet and the possible absence of some of the factors of the vitamin-B group, may be responsible for the high fat content found in the livers and also in the carcasses of the rats receiving the ZTL diet. The reduction in the fat content of the body and the liver which is found on leaving out lysine (ZT group) may be explained by the fact that these animals do not grow at all, and the methionine which is present in a concentration of 2.35% in zein (Baernstein, 1932) is available for choline synthesis. The suggestion that increased growth accentuates the effects of a choline deficiency by rendering less methionine available for choline synthesis, and also by raising the demand for choline, has been put forward by several workers (Griffith & Mulford, 1941; Treadwell, Tidwell & Gast, 1944). On the other hand, Tucker & Eckstein (1938) have found that fatty livers produced by a gliadin diet are not affected by the addition of lysine.

Protein content of the liver and carcass. The protein content of the carcass is approximately the same for the three groups as shown in Table 2. The liver

protein contents, however, differ considerably for the different diets (Table 3). In the SR group the weight of the liver relative to total body weight is reduced; but this is compensated by an increased protein content of the liver in such a way that the amount of liver protein/100 g. body weight is nearly the same as in the ZTL group. It follows, therefore, that restriction of food intake as such does not lead to a reduction of liver protein. The ZTL group shows an increase of liver weight relative to body weight, but this is accounted for by an increased fat content, and if allowance is made for this the relative liver weights are almost the same for the ZT and ZTL groups (Table 3, column 5). The protein content of the liver of the lysine-deficient group is considerably reduced as compared with the two control groups. The reduction is equally great, whether the comparison is carried out on the basis of the amount of liver relative to the dry weight of the liver, or relative to the fresh weight, or on the basis of the ratio of liver protein to body weight. The conclusion can therefore be drawn that lysine deficiency produces a reduction in liver protein which is not due to decreased food intake, as comparison with the SR group clearly demonstrates. Since the content of solids is nearly the same in the livers of all three groups, it follows that the livers of lysine-deficient

animals contain a higher proportion of non-protein matter not extractable by ether, presumably glycogen.

DISCUSSION

The main symptoms produced by lysine deficiency in rats of an initial weight of 100–120 g. are: moderate loss of body weight, a fairly mild anaemia with a slightly greater average reduction in haemoglobin than in cell number, a hypoproteinaemia and a decrease of liver protein. It has been shown that none of these symptoms, apart from loss of weight, is produced by a simple restriction of food intake on a qualitatively adequate diet. The anaemia is mild, compared with an iron-deficient anaemia, and may be due to an inhibition of haemoglobin formation or to an interference with the formation of the structural unit, the red cell. The lysine content of rat haemoglobin is not known, but by analogy with other species it may be assumed that the haemoglobin of the rat contains about 9–10% of lysine, i.e. more than most other proteins in the body. This and the hypochromic character of the anaemia favour slightly the hypothesis that it is the synthesis of haemoglobin which is disturbed. Albanese *et al.* (1943) have recently shown that tryptophan-deficiency produces an anaemia in rats similar to the one described here and it is highly probable that these anaemias are not specific for deficiencies in particular amino-acids, but are the result of an inhibition of formation of protein as a whole. Such an interpretation is supported by the observations of Weech, Wollstein & Goettsch (1937) on dogs. These authors found that a diet very low in protein produces a progressive fall in total circulating haemoglobin. Measurements of haemoglobin concentration per unit volume of blood obscured this decline, since the plasma volume fell more rapidly at first than total haemoglobin. If lysine deficiency in rats also produces such a reduction of plasma volume, the anaemia would be more severe than our figures indicate. This interpretation, which assumes that prolonged deficiency of lysine affects the production of globin, is also in agreement with the work of Hahn & Whipple (1939), who found that the output of new haemoglobin in anaemia due to loss of blood can be kept at low levels by limitation of dietary protein. The results of Pearson, Elvehjem & Hart (1937), who noted that recovery from an iron-deficient anaemia is not greatly influenced by the quality of the protein given during the recovery period, are not in conflict with this conception. In the latter case the protein stores are filled and the limiting factor for growth and haemoglobin formation is the iron intake. If now iron is given, the formation of haemoglobin, which takes precedence over that of plasma proteins, for example (Robscheit-Robbins, Madden, Rowe, Turner & Whipple,

1940), proceeds at the expense of the protein stores of the body. Amino-acid deficiency will therefore only affect the synthesis of haemoglobin when the protein reserves have considerably decreased. Protein has not been much discussed as a causative agent of experimental or clinical anaemia. Recently, however, Orten & Orten (1943) produced a mild, hypochromic anaemia in rats by feeding a diet low in protein but adequate in other respects, and Bethell (1936) reported that the haemoglobin content of the blood of pregnant anaemic women can be raised by an increased protein intake. It appears very probable, therefore, that a deficiency in total protein or particular amino-acids may at least be a contributory factor in the anaemia found in cases of malnutrition.

The reduction of the protein content of the liver and the associated hypoproteinaemia contrast strongly with the apparently normal nitrogen content of the carcass. A progressive fall in serum proteins and a decrease in the protein content and a slight increase in the water content of the liver were also found in dogs fed a diet low in proteins (Elman & Heifetz, 1941). It appears that the liver is particularly sensitive to changes in the amino-acid composition of the diet. This quick reaction of the level of liver protein to dietary changes has already been described by Addis, Poo & Lew (1936); these authors found that the loss of protein resulting from starvation is more pronounced in the liver than in other organs; it occurs at an earlier stage. In a later paper (Addis, Lee, Lew & Poo, 1940) it was shown that the protein content of the liver, kidney and plasma varies considerably with the level of protein in the diet. The effect of changes of dietary protein on the composition of the liver has also recently been examined by Kosterlitz (1944), who found a decrease of liver protein if rats were kept either on protein-free diets or on rations deficient in essential amino-acids like tryptophan. From a simultaneous analysis of the nucleic acid and phospholipid content of the liver the conclusion was drawn that the protein lost is derived from the cytoplasm and that a certain fraction of the cytoplasmic protein is very labile metabolically. The conception of a labile protein store in the liver has also been used to interpret the many observations made by Whipple and his collaborators on the regeneration of plasma protein and haemoglobin after loss of blood or plasma (Madden & Whipple, 1940). Lysine deficiency in the diet apparently reduces the labile protein store in the liver in the same way as a general reduction of protein intake, without affecting greatly the protein content of the body as a whole. The hypoproteinaemia found is best explained by assuming that the liver, and possibly this labile cytoplasmic fraction, is closely concerned with plasma protein formation.

SUMMARY

1. Lysine deficiency in rats of an initial weight of 110–140 g. produces a mild anaemia with a slightly greater reduction in haemoglobin than in red cells. It is suggested that protein deficiency may be a contributory factor in nutritional anaemia.

2. There is also a loss of protein from the liver and a hypoproteinaemia, whilst the body protein content seems to be unchanged. The importance of the liver for protein metabolism and its possible role

in connexion with the synthesis of serum proteins is discussed.

3. None of these symptoms, apart from a loss of body weight, is observed if an adequate diet is restricted in amount to produce weight changes similar to those observed in lysine-deficient animals. It is suggested that the clinical picture is produced by a general interference with protein synthesis and does not represent a specific lesion.

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The Pigments of Sponges

1. THE LIPID PIGMENTS OF THE SPONGE *HYMENIACIDON SANGUINEUM* (GRANT)

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The classification of carotenoid pigments into those present in plants, exhibiting the normal triplet spectrum, and those of animal origin, such as astacene, glycymerin and echinenone, with a single-banded spectrum, would not appear to be correct. Recently Heilbron, Jackson & Jones (1935) and Lederer (1938) have isolated animal xanthophylls possessing three absorption bands, e.g. pectenoxanthin, $C_{40}H_{54}O_3$, pentaxanthin, $C_{40}H_{56}O_5$, and sulcatoxanthin, $C_{40}H_{52}O_8$. It would seem, however, that no corresponding animal hydrocarbons have

so far been identified. Lederer (1938) has made a study of the carotenoid pigments of some fourteen species of invertebrates and has recorded his failure, save in two cases, to isolate any plant xanthophylls, a striking fact when it is remembered that the food of marine animals may include seaweed containing the xanthophylls fucoxanthin, taraxanthin and lutein. In view of this interesting observation of Lederer and in order to obtain further information with regard to the pigments of invertebrates a study has been made of the colouring matters of certain