CLXXXVI. ANIMAL PHENOLASES AND ADRENALINE

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RECENT work on the enzymic oxidation of adrenaline has suggested that the animal phenolases might play a physiological role in the inactivation of adrenaline liberated at adrenergic nerve endings [Green & Richter, 1937; Bacq, 1938]. Since data are available as to the distribution of the chromaffin system in the invertebrates it appeared that a comparative study of the distribution of the phenolases would help to show if there is any relation between the two systems.

Heard & Raper [1933] showed that adrenaline is oxidized by the catechol oxidase of mealworms and Neuberg [1908] described a phenolase in *Sepia* which inactivates adrenaline. The literature contains a number of other references to the occurrence or absence of phenolases in particular animal tissues [Oppenheimer, 1926; Pugh, 1934] but quantitative data as to the amount of enzyme present are lacking and the enzymes are often ill-defined so that it is not clear to what extent they would oxidize adrenaline. The older workers generally used the colour formation on incubating in the air with an oxidizable phenol such as catechol or quinol as a method of testing for phenolases, but in many cases the *p*H was inadequately controlled so that the enzymic nature of the oxidations described appears doubtful. This method is further unreliable since small amounts of reducing substances such as ascorbic acid or glutathione in the tissue preparations may prevent the appearance of colour.

In the present work the manometric method of measuring the oxygen uptake was used. Preliminary experiments were first carried out with tissue preparations obtained from a series of animals of different phyla in order to obtain an approximate value for the amounts of enzymes of the catechol oxidase type which are present. Homocatechol (3:4-dihydroxytoluene) was used for this purpose in preference to catechol as it is more rapidly oxidized by catechol oxidase and is more closely related to adrenaline and the other naturally occurring catechol derivatives, which generally have a carbon chain attached to the catechol nucleus.

Oxidation of homocatechol by animal tissues

The tissue preparations were made by grinding the tissues for 20 min. with sand, adding 2 vol. of *M*/15 phosphate buffer, centrifuging for 5 min. and making the resulting solution up to a known volume with *M*/15 buffer solution. With tissues such as liver which show a rapid spontaneous O_2 uptake the extract was dialysed and 1/10 vol. *M*/1·5 buffer then added before diluting. Buffer of *p*H 7·4 was used except where otherwise stated. With *Aurelia* the extract was buffered with 1/10 vol. *M*/1·5 buffer but was not otherwise diluted. The O_2 uptakes were measured by the Warburg method; each cup contained 2 ml. tissue extract and

0·2 ml. water or $M/4$ homocatechol solution. The homocatechol was prepared from vanillin by reduction and demethylation and had B.P. 135°/12 mm. With tissues containing active enzymes the extract was diluted 1 : 6 or 1 : 12 and the O_2 uptake during the first 4 min. was measured. With the relatively inactive tissues the extract was used undiluted and the O_2 uptake was measured during 1 hr. The O_2 uptakes are given in Table I as μl. O_2/g. fresh tissue or blood/hr. Individual specimens often showed considerable variation, but the figures given nevertheless show the order of magnitude of the amount of phenolase present.

With the *Cancer* and *Helix* preparations the oxidation rate showed a slight autocatalytic increase: other systems showed a falling off in the oxidation rate after the first few minutes, but the oxidation rates remained in general approximately constant during the time of measurement. Since the plant phenolases have been more fully investigated than the animal enzymes four plant tissues were included for comparison; experiments showing the effect of adding 10^{-3} M HCN are also given.

In these preliminary experiments no attempt was made to study the properties of the purified enzymes but rather to observe their activity in the presence of the other cell constituents. The purified catechol oxidases are comparatively labile and are rapidly inactivated by the reaction products formed in the oxidation of catechol derivatives [Richter, 1934], but the inactivation is much less rapid when other tissue proteins are present. Many of the extracts showed a red colour during the oxidation, but in some cases the colour was masked by that of the extracts themselves. The small O_2 uptakes, of the order of 100 μl. O_2/g./hr., found for many of the tissues in the presence of homocatechol may be ascribed in part to non-enzymic catalysis by traces of heavy metals and in part to the cytochrome oxidase system which catalyses the slow oxidation of catechol derivatives [Green & Richter, 1937; Keilin & Hartree, 1938, 1]. The large O_2 uptakes, of 3000 or more, observed in several of the arthropods must be attributed to the very active catechol oxidases (also described as "tyrosinases" or "polyphenol oxidases") which are known to occur in animals of this phylum.

In the insect larvae the catechol oxidase is present in the blood but is apparently not confined to the blood since it was found in similar or even higher concentration in the skins which were carefully freed from blood by drying on filter paper, washing with water and pressing on filter paper again. The blood was obtained free from contamination by other tissues by holding the insects firmly between the finger and thumb so as to force the blood towards the middle and then making an incision in the skin.

The vertebrate tissues tested were found to be comparatively inactive, especially in view of the fact that the estimations were done at 37° while 25° was used for most of the other animals. Apart from the arthropods relatively high activity was exhibited only by certain molluscs in which O_2 uptakes of 300–500 μl./g./hr. were obtained. This observation of high activity in the arthropods and molluscs appeared specially suggestive in view of the recent work of Kubowitz [1937] and of Keilin & Mann [1938] who have shown that certain plant phenolases are copper-protein complexes, for it is precisely in the arthropods and molluscs that the copper-containing haemocyanins occur. The systems responsible for the oxidation of catechol derivatives in these two phyla were therefore selected for study in greater detail: the crab *Cancer pagurus* and the edible snail *Helix pomatia* served as convenient sources of material.

Table I. *Rate of oxidation of homocatechol*

Phylum	Species	Tissue	Temp. °C.	μl. O_2/g. tissue/hr. Tissue alone	With substrate	Difference
Coelenterata	*Actinia equina* L.	Whole animal	26	0	69	69
	,,	Whole animal (+HCN)	26	9	61	52
	Tealia felina (L.)	Muscle	24	15	70	55
	,,	Muscle (+HCN)	24	9	25	16
	,,	Mesenterium	26	60	48	−12
	,,	Mesenterium (+HCN)	26	39	32	−7
	Aurelia aurita (L.)	Denser parts	25	0	2	2
Nematoda	*Ascaris lumbricoides* var. *Suis*	Whole animal (*p*H 7·0)	37	51	100	49
Annelida	*Hirudo medicinalis*	Whole animal (*p*H 7·0)	25	4	130	126
	Lumbricus terrestris	Whole animal (*p*H 7·0)	25	0	55	55
Arthropoda	*Odonestis potatoria* (larva)	Blood	25	0	5340	5340
	,, ,,	Skin	25	0	3320	3320
	Lasiocampa quercifolia (larva)	Blood	25	0	623	623
	,, ,,	Skin	25	0	3680	3680
	,, ,,	Viscera	25	64	1065	1001
	Bombyx quercus (larva)	Blood	25	0	3310	3310
	,, ,,	Skin	25	0	8680	8680
	,, ,,	Viscera	25	278	3060	2782
	Eumorpha elpenor (pupa)	Blood	25	0	5130	5130
	,, ,,	Viscera	25	34	3610	3576
	Locusta migratoria (adult: migratory phase)	Viscera	25	494	2440	1946
	Schistocerca gregaria F. (adult: migratory phase)	Viscera	25	192	1105	913
	Dysdercus intermedius Dist. (adult)	Abdomen	25	239	5080	4841
	Periplaneta americana (adult)	Viscera	25	0	141	141
	Cancer pagurus L.	Blood (*p*H 7·0)	30	18	462	444
	,,	Gills (*p*H 7·0)	30	9	256	247
	,,	Hepatopancreas (*p*H 7·0)	30	27	327	300
	,,	Leucocytes (*p*H 7·0)	30	0	4530	4530
	Homarus vulgaris	Blood (*p*H 7·0)	30	7	290	283
Mollusca	*Helix aspersa*	Viscera	25	23	469	446
	Helix pomatia	Blood	30	35	306	271
	,,	Hepatopancreas	30	48	120	72
	Patella vulgata L.	Viscera	24	35	84	49
	,,	Viscera (+HCN)	24	38	75	39
Echinoderma	*Echinus esculentus* L.	Intestine	26	9	56	47
	,,	Intestine (+HCN)	26	7	56	49
	,,	Gonads	26	0	0	0
	Asterias rubens L.	Hepatic caecum	30	7	117	110
	,,	Hepatic caecum (+HCN)	30	5	102	97
Vertebrata	*Rana temporaria*	Liver	37	18	255	237
	,,	Intestine	37	110	107	−3
	Guinea pig	Liver	37	27	197	170
	,,	Intestine	37	7	54	47
	Pig	Kidney	37	72	107	35
	,,	Liver (*p*H 7·0)	37	0	129	129
	,,	Pancreas (*p*H 7·0)	37	6	79	76
	,,	Heart (*p*H 7·0)	37	6	116	110
	,,	Intestine (*p*H 7·0)	37	10	66	56
	,,	Spleen (*p*H 7·0)	37	24	51	27
	Sheep	Liver (*p*H 7·0)	37	11	156	145
	,,	Pancreas (*p*H 7·0)	37	14	87	73
	,,	Kidney (*p*H 7·0)	37	12	194	183
	,,	Heart (*p*H 7·0)	37	6	121	115
	,,	Intestine (*p*H 7·0)	37	6	74	68
	,,	Spleen (*p*H 7·0)	37	0	162	162
	,,	Brain (*p*H 7·0)	37	7	29	23
	,,	Thyroid (*p*H 7·0)	37	0	111	111
	Ox	Liver (*p*H 7·0)	37	17	114	97
	,,	Intestine (*p*H 7·0)	37	30	126	96
Plants	*Agaricus campestris*	Basidium	25	35	>4500	>4465
	Cichorium intybus	Leaf	25	5	>4500	>4495
	Ricinus communis	Seed	25	3	39	36
	Solanum tuberosum	Tuber	25	7	>4500	>4493

Catalytic systems of Cancer pagurus

Haemolymph system. A system that gave rapid colour formation with catechol or homocatechol and O_2 uptakes of 300–800 μl./g./hr. was present in *Cancer* blood or haemolymph. The activity varied considerably from one animal to another. Gills and hepatopancreas were less active than the blood.

	μl./g./hr.		
	Gills	Hepato-pancreas	Blood
Catechol	186	122	795
Homocatechol	153	300	444

Catechol and homocatechol were used for testing the activity of the preparations and the O_2 uptakes were measured at *p*H 7·0 and 30°; otherwise the conditions were the same as are described above. The activity showed a marked increase on dialysis: two specimens of blood gave with homocatechol (*a*) undialysed, 284, 444, and (*b*) dialysed, 445, 1180 μl./g./hr. This is apparently due to an inhibitor which is removed by dialysis. Similar systems were also found in the blood of *Homarus vulgaris*, *Helix aspersa* and the squid *Loligo*, and similar figures were obtained with dialysed and undialysed specimens. The system present in *Cancer* blood had the properties of an enzyme in that it was inactivated by boiling, by digestion with trypsin, by drying or by treating with 95 % alcohol.

Purification. An active preparation was obtained from *Cancer* blood as follows. The animals were cooled to 0° by leaving for a few hours in the ice chest. The blood was obtained by puncturing the heart or severing the dorsal artery and was run directly into centrifuge cups cooled to 0° in ice: each animal gave 50–75 ml. blood. The leucocytes were centrifuged off and the clear liquid was dialysed overnight against distilled water at 0°. The precipitate of protein containing carotenoid pigments which separated was inactive and was centrifuged off. The clear solution containing haemocyanin darkened slowly on keeping but remained unchanged in catalytic activity for several weeks when kept at 0°. Attempts to purify the active system further by precipitation with ammonium sulphate, alcohol or acetone gave rise to gelatinous products which could not readily be centrifuged or filtered.

The catalytic system appeared to be closely associated with the haemocyanin fraction of the blood proteins. Attempts were therefore made to purify the system further by the methods used for crystallizing haemocyanins. To the clear solution obtained by dialysis as described above 5 % acetic acid was added drop by drop with stirring until the solution showed a slight permanent turbidity and gave an orange colour with methyl red (*p*H 4·7). On dialysing again against distilled water at 0° the protein separated out in the form of a colourless crystalline precipitate. The crystals were small but could be seen to be in the form of hexagonal plates when viewed under the microscope.

The crystals slowly turned a pale greenish blue at the surface on exposure to air. They were insoluble in water but dissolved rapidly and completely in dilute salt solutions. A crystalline protein has not previously been obtained from the blood of *Cancer pagurus*. The protein described, while resembling a haemocyanin in its ready solubility in dilute salt solutions, differed from the haemocyanins in being colourless. The aqueous solution showed a very pale greenish colour but did not turn blue or give a measurable O_2 uptake on shaking for 5 min. with air. Copper estimations showed that the protein contained about the right amount of copper for a haemocyanin (Found: Cu, 0·13, 0·12 %). The protein

showed several points of resemblance to the methaemocyanin obtained by Conant *et al.* [1933] by treating haemocyanins with oxidizing agents: it is apparently a modified form of haemocyanin produced during the process of crystallization. The purified crystalline protein showed considerable catalytic activity when tested in the usual way with homocatechol, the activity corresponding to that originally present in the blood: the mother liquor from which the crystals were obtained showed practically no activity. The activity also remained constant on recrystallizing.

Table II. *Catalysis by crystalline copper-protein complex*

	Dialysed blood (33·3 mg./2 ml.)		Crystalline prep. (28·6 mg./2 ml.)	
	μl. O_2/hr.	Q_{O_2}	μl. O_2/hr.	Q_{O_2}
Catechol	415	12·5	466	16·3
Homocatechol	170	5·1	239	8·3
Adrenaline	4	0·1	12	0·4

Although the purified *Cancer* preparation was very active when tested with catechol and homocatechol it was found, surprisingly, that it showed comparatively little activity with adrenaline as substrate. The catalytic activity was strongly inhibited by cyanide and also by the copper reagent cupferron. The inhibitions found at a concentration of 1/2000 inhibitor in the oxidation of catechol under the conditions previously described were: HCN, 95 %; NaF, 43 %; cupferron, 69 %; sodium diethyldithiocarbamate, 9 %; Na_2S, 24 %; 8-hydroxyquinoline, 29 %. The activity of the preparation was not appreciably changed by treating with dilute acetic acid (*p*H 3·0) for 4 hr. Under these conditions the haemocyanin is known to undergo modification so that the copper, although it is still combined and cannot be removed by dialysis, becomes capable of catalysing the oxidation of thiol derivatives [Pirie, 1931]. In the crystalline preparation the dialysis at *p*H 4·7 may have already brought about this modification.

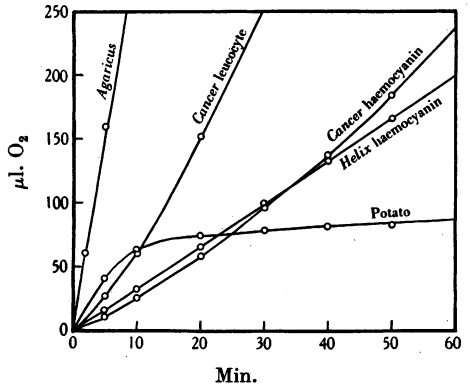

Fig. 1. Oxidation of homocatechol. Conditions as given for Table I. Protein content of preparations in 2·2 ml.: *Agaricus*, 1·7 mg.; *Cancer* leucocyte, 5·9 mg.; *Cancer* haemocyanin, 28·6 mg.; *Helix pomatia* crystalline haemocyanin, 12·0; potato, 0·6 mg.

Cancer *leucocyte catechol oxidase*. Experiments with crude extracts of gills and hepatopancreas showed that these extracts, unlike the purified preparations from centrifuged blood, catalysed the oxidation of adrenaline. This indicated

the presence of a second catalytic system, which was found in highest concentration in the leucocytes. Pinhey [1930] concluded from colorimetric experiments that crustacean bloods contain a phenolase which is liberated by the bursting of the "explosive corpuscles" of Hardy. In the present investigation Pinhey's observations were confirmed.

The leucocytes were separated from the blood by centrifuging at 0°, washing rapidly with saline and centrifuging again. The extract prepared by grinding with sand in the usual manner contained a very powerful phenolase which catalysed the oxidation of adrenaline as well as that of catechol and homocatechol; the extract also catalysed the oxidation of monohydric phenols such as *p*-cresol.

	μl. O_2/g. tissue/hr.	Q_{O_2} of preparation (μl./mg. dry protein/hr.)
Catechol	2820	49
Homocatechol	4830	83
Adrenaline	5840	100

The Q_{O_2} values obtained for the leucocyte preparations were much higher than with the blood plasma preparations. The enzyme present was similar in activity and specificity to the well-known mealworm catechol oxidase.

Catalytic system of Helix pomatia

In the snail, as in the crab and lobster, the blood contains a system that gives colour formation and O_2 uptake with catechol or homocatechol. Here again the system was closely associated with the haemocyanin fraction of the blood proteins. Crystalline haemocyanin prepared by the method of Dhéré [1919] showed a considerable catalytic activity corresponding to that of the blood. The system was relatively inactive with adrenaline as substrate.

	Dialysed *Helix* blood (14·6 mg. protein in 2 ml.)		Crystalline haemocyanin (12 mg. protein in 2 ml.)	
	μl. O_2/hr.	Q_{O_2}	μl. O_2/hr.	Q_{O_2}
Catechol	160	10·9	134	11·2
Homocatechol	196	13·4	176	14·6
Adrenaline	25	1·7	30	2·5

Catalysis by heavy metals

That traces of heavy metals can catalyse the oxidation of phenols is well known (a good review of the literature is given by Sutter [1936]). In view of their similarity to the plant enzymes the animal catechol oxidases are probably also Cu-protein complexes. The observed catalytic activity of the haemocyanin preparations raises the question of how far the activity of the other phenolases described in the literature may be ascribed to the specific or unspecific catalysis by heavy metals or heavy metal-protein complexes. The activity of the haemocyanin preparations might be attributed to traces of loosely combined Cu present as an impurity in the preparations. It therefore appeared desirable to determine the catalytic activity of traces of heavy metals under the conditions used throughout and to check this against the actual heavy metal content of the preparations.

For several of the substrates tested there was a large increase in the oxidation rate as shown by the colour formation and O_2 uptake on adding Cu, Ni or Co at concentrations of the order of 0·1 mg. metal/ml. Of the commoner heavy metals tested Fe was comparatively inactive as a catalyst; Cu was the most

Table III

O_2 uptakes (μl./hr.) in the presence of heavy metals at *p*H 7·0 and 30°. Each Warburg vessel contained 0·2 mg. metal in 2 ml. *M*/15 phosphate buffer and 0·2 ml. *M*/4 substrate. The metals were added in the form of the sulphates.

Substrate	No addition	Cu	Fe	Ni	Co	Mn
Catechol	2	45	12	119	65	13
Homocatechol	34	238	59	313	163	59
Adrenaline	6	115	55	266	251	2
Guaiacol	0	0	0	2	4	0
Protocatechuic acid	2	0	0	8	19	0
Pyrogallol	82	115	42	286	218	76
Phloroglucinol	0	3	0	3	16	3
p-Cresol	0	0	0	0	6	0
Quinol	4	18	2	8	12	20
p-Phenylenediamine	0	198	0	0	135	0

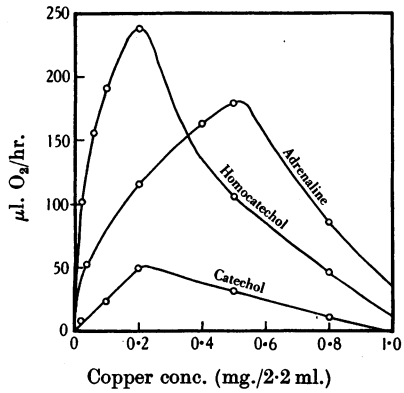

Fig. 2. Effect of Cu concentration on the rate of oxidation. The manometers contained 2 ml. *M*/15 phosphate buffer of *p*H 7·0 and 0·2 ml. *M*/4 substrate. Experiments at 30°.

active of those present in significant amounts in most animal tissues. Using Cu as a catalyst the rate of oxidation increased with the Cu concentration (Fig. 2) until high concentrations were reached at which the amount of Cu present was comparable with the amount of phenol, when there was a falling off in oxidation rate. The effect of adding gelatin to the system to form the copper-gelatin complex depended on the substrate and on the Cu concentration, but no very large increase in catalytic activity was observed. The gelatin used was purified by the method of Northrop & Kunitz [1927].

Table IV

O_2 uptakes (μl./hr.) at different Cu concentrations, with and without gelatin. Conditions as in Table III. The figures given for the Cu concentration represent mg. Cu in each Warburg vessel (total vol. 2·2 ml.). The series marked "Gel." contained 7 mg. gelatin.

	Cu concentration											
	0·02		0·05		0·1		0·2		0·5		1·0	
Substrate	—	Gel.	—	Gel.	—	Gel.	—	Gel.	—	Gel.	—	Gel.
Homocatechol	102	76	149	100	193	135	238	190	108	177	15	109
Catechol	10	25	14	31	20	38	60	63	34	71	0	38
Adrenaline	46	115	—	—	—	—	115	190	182	225	37	154
Pyrogallol	92	92	—	—	—	—	115	116	133	165	126	220

Although the catalytic activity of Cu is very considerable these experiments show that the activities of the haemocyanin and leucocyte systems are much greater than that of an equivalent amount of inorganic Cu: their activities cannot therefore be due to small amounts of Cu present as an impurity in the preparations. Inorganic Cu is also much less specific in that it catalyses the oxidation of *p*-phenylenediamine, for example, which is not oxidized by the haemocyanin system or the catechol oxidases. This is shown in Table V in which the specificities of a number of phenolases and *pseudo*phenolases are compared.

Table V. *Specificity of catalytic systems*

O_2 uptakes (μl. O_2/2 ml./hr.) at *p*H 7·0 and 30°. Conditions as for Table I.

	Cancer haemocyanin	*Helix* haemocyanin	*Cancer* leucocyte	Potato	*Agaricus*	*Dolichos*	Cu	Buffer only
mg. protein/2 ml.	27·3	14·6	5·9	0·6	0·3	3·3	0	0
γ-Copper/2 ml.	32·8	32·2	6·5	0·5	0·4	0·3	200	<0·1
Catechol	466	160	286	176	393	229	45	2
Homocatechol	189	196	490	415	385	139	238	34
Adrenaline	21	25	591	210	365	334	115	6
Protocatechuic acid	0	2	3	16	1	5	0	2
Guaiacol	5	0	5	—	1	—	0	0
p-Cresol	2	9	742	248	189	0	0	0
Phloroglucinol	2	5	0	72	1	3	3	0
Quinol	12	19	12	36	61	20	18	4
Pyrogallol	231	122	260	185	263	76	115	82
p-Phenylenediamine	12	7	0	9	1	10	198	0

The enzymes were prepared from the mushroom *Agaricus campestris* (basidium), the legume *Dolichos* (seed) and potato tuber by grinding the tissue with sand, extracting with buffer of *p*H 7·0, precipitating with alcohol and dialysing. The Cu estimations which are included in Table V were done colorimetrically with sodium diethyldithiocarbamate.

The large differences in the specificity of the systems towards different substrates are very striking, and are sufficiently well-defined to be used for identifying them. The *Cancer* leucocyte enzyme shows a specificity similar to those of the mushroom and potato enzymes. Inorganic Cu differs both from the phenolases and from the haemocyanin systems in specificity, but in catalysing the oxidation of *p*-phenylenediamine it resembles the cytochrome system: on other grounds there is evidence that the cytochrome oxidase is a Cu-protein complex [Keilin & Hartree, 1938, 2].

Discussion

(*a*) *Catalytic activity of haemocyanins.* Catechol and homocatechol gave rapid O_2 uptakes accompanied by colour formation when shaken with the blood of species of *Cancer*, *Homarus*, *Loligo* and *Helix*. The natural inference was that this was due to phenolases in the blood, and this appeared to be confirmed when the systems were found to be thermolabile and to show other enzymic properties. Attempts to purify the phenolases then led to the preparation of purified specimens of haemocyanin or Cu-protein complexes derived from the haemocyanins, one of which was obtained crystalline. Crystalline *Helix* haemocyanin also showed considerable phenolase activity. It appeared possible that the catalytic activity might be due to traces of very active phenolase present as an impurity, but this became improbable when it was found that the activity of the haemocyanins remained constant on recrystallization and was higher than that of the unpurified blood proteins. It could be shown that the catalytic activity of the haemocyanins was not due to inorganic Cu contained in the preparations

since an equivalent amount of Cu was much less active and less specific as a catalyst than when combined in the form of haemocyanin. It is therefore concluded that the haemocyanins are themselves catalytically active and can act as *pseudo*phenolases. This *pseudo*phenolase activity of the haemocyanins is comparable with the *pseudo*peroxidase activity of the haemoglobins.

Experiments on the mechanism of the catalysis indicated that a modified form of haemocyanin, possibly methaemocyanin formed during the experiment by the action of traces of *ortho*quinones, is more active than haemocyanin itself since (*a*) the oxidation curves were autocatalytic in shape, (*b*) the modified form of haemocyanin produced by treatment with acid was equally active and (*c*) adrenaline, the *ortho*quinone of which is very labile, was not appreciably oxidized.

The catalytic activity of the haemocyanins is much lower than that of the catechol oxidases, but it is sufficient to give a strongly positive phenolase test by the colorimetric method and to account for the apparent phenolase activities of a number of arthropod and mollusc tissues.

(*b*) *Distribution of phenolases.* Catechol oxidases are present in a number of arthropods, including the crab *Cancer pagurus* where the enzyme occurs chiefly in the leucocytes. Active phenolases of this type were not found in animals of any other phylum. The number of animals tested is small, but in those hitherto tested apart from the arthropods the results show that if any catechol oxidase is present the amount must be comparatively small.

The literature contains many references to the existence of phenolases in other animal phyla [Oppenheimer, 1926], but the colorimetric technique has generally been used for showing their presence. By the colorimetric method the general practice has been to incubate the tissue extracts at alkaline reaction and 37° with an oxidizable phenol such as "dopa" and examine the extracts after 12–24 hr. for colour formation [Bloch, 1917; Pugh, 1933]. Colour formation under these conditions is an extremely sensitive test which may be given by a number of different types of catalysts. A phenolase which is so inactive that it can be detected only in this way must have quite a different order of activity from the arthropod or plant enzymes, which give high Q_{O_2} values and a coloration with homocatechol at *p*H 7·0 and 25° in less than a minute.

The present observations on the *pseudo*phenolase activity of Cu-protein complexes show that colour formation in the presence of an oxidizable phenol, even when supported by evidence of thermolability, cannot be taken as sufficient evidence of the existence of a phenolase. Thermolability is unreliable as a criterion of the enzymic nature of a phenolase as small amounts of heavy metals may remain adsorbed on the protein and so may be removed from solution when a protein is coagulated: Stotz *et al.* [1937] showed that artificially prepared Cu-protein complexes resemble enzymes in their thermolability. Cu is present in many animal tissues in sufficient amounts to catalyse the slow oxidation of catechol derivatives; Cunningham [1931] found as much as 0·1–0·9 mg. Cu/g. in a number of animal tissues while 0·025 mg. Cu/g. is sufficient to give a positive colorimetric "phenolase" test with "dopa".

It has recently been shown that the cytochrome oxidase system which is present in many animal tissues can also catalyse the oxidation of catechol derivatives [Green & Richter, 1937; Keilin & Hartree, 1938, 1]. In view of these observations the enzymic nature of a number of the phenolases described in the literature would appear to be doubtful.

(*c*) *Physiological function of phenolases.* The available evidence gives no support to the view that the catechol oxidases are in any way related to the

chromaffin system in their function. The highest phenolase activity was found in the arthropods, in which no chromaffin system has been found, while *Lumbricus* and *Hirudo* which have well-defined chromaffin systems showed little phenolase activity.

There seems no reason to believe that the *pseudo*phenolase activity of the haemocyanins is of any physiological significance. The very active phenolase of *Cancer* leucocytes, on the other hand, may be expected to come into action by the disintegration of the corpuscles when bleeding owing to an injury occurs [Pinhey, 1930]: the rapid darkening of the shed blood gives evidence of the activity of the liberated phenolase and this is seen particularly clearly with *Maia squinado* and many insects in which the blood rapidly turns nearly black. The physiological function of the phenolases may perhaps be sought in the properties of the *ortho*quinones to which they give rise, such as their activity as respiratory carriers or their bactericidal action.

Summary

1. A number of animal tissues have been examined for the presence of phenolases of the catechol oxidase type.

2. A crystalline Cu-protein complex which is catalytically active has been obtained from the blood of *Cancer pagurus*.

3. Haemocyanins and other Cu-protein complexes can act as *pseudo*phenolases: they are mainly responsible for the apparent phenolase activity in a number of arthropods and molluscs.

4. The catalytic activities of Cu, Fe, Co, Ni and Mn in the oxidation of a series of phenols have been examined.

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