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Some Observations on the Reaction of Catechol Derivatives with Amines and Amino Acids in Presence of Oxidizing Agents

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The hardening of the cockroach ootheca, which is protein in nature and contains no chitin, is brought about by interaction of a phenolic substance secreted by the right colleterial gland of the female insect with protein secreted by the left gland in the presence of an oxidase system (Pryor, 1940a). It was suggested on the basis of this and other evidence that the hardening of the exocuticle of insects is due to the tanning of the protein constituent by an agent produced by oxidation of a phenolic substance (Pryor, 1940b). Later, the phenolic substance responsible for the hardening of the cockroach ootheca was identified as 3:4-dihydroxybenzoic acid (protocatechuic acid) (Pryor, Russell & Todd, 1946), and the presence of the same acid, or of the related 3:4-dihydroxyphenylacetic and 3:4-dihydroxyphenyllactic acids, in a number of other insect species was confirmed (Pryor, Russell & Todd, 1947; Hackman, Pryor & Todd, 1948). The actual hardening process envisaged involved oxidation of one or other of these *o*-dihydroxy acids to an *o*-quinone, followed by condensation of the latter with the —NH_2 (and possibly also —SH) groups in the protein molecules to give a stable, cross-linked structure in which the nitrogen of the free amino groups was directly attached to the aromatic nucleus. The effect of this condensation on the cuticular protein would be to reduce the solubility and increase resistance to attack by hydrolytic

agents. The process would be indeed analogous to the tanning of collagen by *p*-benzoquinone, which has been used in the production of leather.

With the object of throwing further light on the mechanism of the hardening process, we have carried out some experiments on the oxidation of various catechol derivatives in the presence of amines and amino acids, using both chemical and enzymic methods. Further, as it is considered that the nitrogen of the amino groups becomes directly attached to the quinonoid nucleus, compounds related to *N*-(3:4-dioxocyclohexa-1:5-dienyl)aminoacetic acid (VII) have been synthesized and their properties investigated.

The reaction between *o*-benzoquinone derivatives and compounds containing amino groups has not been extensively studied in the past. Kehrman & Cordone (1913) reported that oxidation of catechol in acetic acid solution in presence of excess aniline gave 4:5-dianilino-1:2-benzoquinone (I), and Pugh & Raper (1927) obtained the same compound by aeration of a buffered solution of phenol or catechol and aniline containing the enzyme tyrosinase; from homocatechol, *m*- or *p*-cresol, under similar conditions, they obtained 'dianilinohomoquinoneanil'. They also prepared (I) directly from *o*-benzoquinone and aniline, although, much earlier, Jackson & Koch (1898) prepared an aqueous solution of *o*-benzoquinone by oxidizing the lead salt of catechol

with iodine, and, by treating the crude quinone in chloroform solution with aniline, obtained 4:5-dianilino-1:2-benzoquinoneanil (II). Apart from the work of Chodat (1912), who described the development of various colours on treating *o*-benzoquinone with compounds containing amino groups, these appeared to be the only relevant investigations in the literature at the time the present work was undertaken. Since then, Jackson & Kendal (1949) have reported the preparation of 4-(2-ethoxycarbonyl-4-hydroxy-1-pyrrolidyl)5-methyl-1:2-benzoquinone from 4-methylcatechol and hydroxyproline ethyl ester hydrochloride, and Trautner & Roberts (1950) have proposed a mechanism for the oxidative deamination of amino acids by catechol and polyphenolases which, for glycine, includes as an intermediate the compound *N*-(3:4-dioxocyclohexa-1:5-dienyl)aminoacetic acid (VII). The same intermediate has been demonstrated in polarographic studies by Dorskocil (1950).

It was decided first to examine the reaction of *o*-benzoquinone derivatives with aniline as a pre-

liminary to experiments using amino acids, because it was expected that the products obtained would be more readily identified, and the basic reactions involved would be similar in the two cases. Enzymic and non-enzymic methods were employed for the oxidation of the catechol derivatives. In the enzymic method a solution of the catechol derivative in phosphate buffer (pH 6.7) containing aniline and polyphenol oxidase was aerated for 48 hr.; the precipitated condensation product was then separated and purified. The non-enzymic procedure was a modification of that used by Kehrmann & Cordone (1913), in which the catechol derivative was oxidized in glacial acetic acid in presence of aniline using silver oxide or silver acetate, the product being precipitated by dilution with water. The results obtained by the two methods are given in Table 1.

The structures of the products obtained by either method of oxidation from 4-methylcatechol and 2-(3:4-dihydroxyphenyl)propionic acid are admittedly unproven, but the most probable formulae would appear to be (IV) and (V). The structure of

Table 1. *Oxidation of catechol derivatives in the presence of aniline*

Substance	Product of oxidation	
	Enzymic	Non-enzymic
Catechol	4:5-Dianilino-1:2-benzoquinone (I)	4:5-Dianilino-1:2-benzoquinone (I)
3:4-Dihydroxybenzoic acid	4:5-Dianilino-1:2-benzoquinone (I)	4:5-Dianilino-1:2-benzoquinoneanil (II)
3-Methylcatechol	4:5-Dianilino-3-methyl-1:2-benzoquinone (III)	4:5-Dianilino-3-methyl-1:2-benzoquinoneanil (VI)
4-Methylcatechol	Dianilino-4-methyl-1:2-benzoquinoneanil (IV)	Dianilino-4-methyl-1:2-benzoquinoneanil (IV)
2-(3:4-Dihydroxyphenyl)-propionic acid	2-(Dianilino-1:2-benzoquinoneanil)-propionanilide (V)	2-(Dianilino-1:2-benzoquinoneanil)-propionanilide (V)

I

II

III

IV

V

VI

VII

VIII

IX

the product (VI) from 3-methylcatechol follows from its identity with 4:5-dianilino-3-methyl-1:2-benzoquinoneanil prepared from 2-hydroxy-5-nitrosotoluene or 3:6-dianilino-2-methyl-1:4-benzoquinone (Zincke, 1883; Fischer & Hepp, 1888). Compounds (I)–(VI) can, of course, be written in several tautomeric forms in some of which they appear as *p*-quinone derivatives; (II), for example, is identical with one product of the reaction between *p*-benzoquinone and aniline. It will be observed that the oxidation and condensation of 3:4-dihydroxybenzoic acid was accompanied by loss of carbon dioxide; this was not unexpected in view of the known tendency of this acid to undergo decarboxylation on warming its alkaline solution, or in presence of warm ferric chloride. It is interesting to note that, although both catechol and 3:4-dihydroxybenzoic acid gave the same product (I) by the enzymic oxidation, 3:4-dihydroxybenzoic acid gave the anil (II) by the non-enzymic method. It is, however, clear that in every case, including that of the insect product 3:4-dihydroxybenzoic acid, oxidation in presence of aniline gave products in which aniline residues were attached directly through nitrogen to the aromatic nucleus.

When similar experiments were carried out in which catechol derivatives were oxidized in presence of amino acids, the situation was more complex and the results were much less satisfactory. On aerating at pH 6–7 in presence of polyphenol oxidase a solution of catechol and various amino acids (glycine, alanine, leucine, phenylalanine, tyrosine or glycylglycine), the solution became first red then reddish violet and no insoluble material separated. The actual amount of coloured product was probably small, and in no case was it isolated in solid form. No doubt most of the amino acid was removed from reaction by oxidative deamination (Strecker degradation) brought about by the *o*-benzoquinone. This oxidative deamination occurs readily with α -amino acids, less so with dipeptides, and with higher peptides it does not occur appreciably (Kisch, 1932). Substitution of β -alanine, aliphatic amines or ammonia in these experiments with catechol led to the formation of resinous products. Very similar results were obtained when the catechol in these experiments was replaced by 3:4-dihydroxybenzoic acid, ethyl 3:4-dihydroxybenzoate, 3:4-dihydroxyphenylacetic acid and 2-(3':4'-dihydroxyphenyl)propionic acid.

Somewhat different results were obtained by non-enzymic oxidation of the various catechol derivatives in presence of α -amino acids. All the amino acids yielded lustrous black solids in the experiments with catechol. These solids were insoluble in water, very sparingly soluble in benzene or chloroform, soluble in ethanol, and very soluble in acetone, acetic acid or pyridine, giving red

solutions. From analytical data and from their general behaviour it appeared that these products were complex and probably polymeric, their composition depending on the experimental conditions employed; thus, under two different sets of conditions using glycine, products were obtained which were made up of catechol and glycine residues in the ratios 3/2 and 9/2, linkage having been effected by loss of water and hydrogen from the starting materials. Attempts to condense solutions of preformed *o*-benzoquinone with α -amino acids yielded similar products of an indefinite nature.

Proteins react readily with quinones (cf. the tanning of collagen). In the presence of polyphenol oxidase aqueous solutions of catechol, 3:4-dihydroxybenzoic acid, 3:4-dihydroxyphenylacetic acid or 3:4-dihydroxyphenylalanine (α -amino- β -(3':4'-dihydroxyphenyl)-propionic acid) reacted with the water-soluble cuticular protein from *Sphinx ligustri* larvae, so as to render it insoluble in water. Tetra-bromophthalein ethyl ester and ninhydrin tests showed the absence of protein in the residual aqueous solutions. When 3:4-dihydroxyphenylalanine was used, the insoluble material was black (probably due to melanin formation), but with all the other compounds it was red. In these cases the cuticular protein was rendered water insoluble by the oxidized polyphenols, presumably by reaction of the latter with the amino groups of the protein chains.

To obtain further information about the properties of products formed in the reaction of an *o*-quinone with a protein, compounds related to *N*-(3:4-dioxocyclohexa-1:5-dienyl)aminoacetic acid (VII) have been synthesized. Compounds of this type should bear a structural relation to the initial condensates obtained from *o*-quinones and amino acids.

Initially, attempts were made to prepare *N*-(3:4-dihydroxyphenyl)aminoacetic acid (VIII; R=H) by acid hydrolysis of *N*-(3:4-dimethoxyphenyl)*N*-toluene-*p*-sulphonamidoacetic acid (IX), since the diacetate of 4-aminocatechol did not react with chloroacetic acid or its esters without decomposition. The acid (IX) was prepared by condensing 3:4-dimethoxy-*N*-toluene-*p*-sulphonanilide with chloroacetic acid or, better, as its potassium salt with ethyl chloroacetate followed by alkaline hydrolysis, but we were unable to effect removal of the toluene-*p*-sulphonyl or methyl groups without gross decomposition. A similar lack of success attended efforts to demethylate *N*-(3:4-dimethoxyphenyl)aminoacetic acid (VIII; R=CH₃). The analogous *N*-(3:4-dibenzoyloxyphenyl)aminoacetic acid (VIII; R=CH₂Ph) similarly decomposed on acid hydrolysis, but its ethyl ester on hydrogenation using a palladized charcoal catalyst gave ethyl *N*-(3:4-dihydroxyphenyl)aminoacetate, further

characterized as its crystalline triacetyl derivative. Direct hydrogenation of (VIII; $R = CH_2Ph$) doubtless yielded the dihydroxy acid (VIII; $R = H$), but the latter could not be isolated, as its solutions rapidly turned yellow and developed a green fluorescence; on working up such solutions only red gummy materials could be obtained.

Although stable in the solid state, ethyl *N*-(3:4-dihydroxyphenyl)aminoacetate hydrobromide underwent spontaneous chemical change in solution. Dissolved in methanol, it rapidly yielded a yellow solution *A* with a powerful green fluorescence, the change being greatly accelerated by heating or by irradiation with ultraviolet light. The appearance of fluorescence strongly suggests formation of a condensed ring system and the solution *A* showed absorption maxima at 410 $m\mu$. and in the region 260–290 $m\mu$. A sample of 3:5:6-trihydroxy-*N*-methylindole (prepared *in situ* by hydrolysing its triacetate) was yellow in methanolic solution, showed a strong green fluorescence and had absorption maxima at 410 and 292 $m\mu$. (cf. Harley-Mason, 1950). In aqueous solution, ethyl *N*-(3:4-dihydroxyphenyl)aminoacetate hydrobromide soon acquired a red colour, apparently due to the formation of a product *B*, which could be more readily obtained by oxidizing the ester in aqueous or methanolic solution with silver oxide. Solutions of *B* thus obtained were deep red in colour and showed absorption maxima at 270, 397 and 430 $m\mu$; reduction with sodium dithionite removed the maximum at 430 $m\mu$, but the solution remained coloured. The oxidized solutions of *B* were not stable, and after 24 hr. they had deposited a deep-violet pigment *C*. This pigment was soluble in water to give a violet solution (absorption maxima 275, 318, 552, 760 $m\mu$). It was amphoteric, giving a blue-green colour with sodium hydroxide and changing through purple and blue to blue-green with acid; the colour changes were reversed in each case by neutralization. In alkaline solution, the pigment *C* was reduced by sodium dithionite giving a yellow solution which was rapidly re-oxidized in air. Molecular weight determinations indicated that *C* was a polymeric substance.

Although the structure of these various products has not been definitely established, it seems that solution *A* probably contained 3:5:6-trihydroxyindole formed from the original ester by elimination of ethanol. The red solution *B* almost certainly owes its colour to the presence of the quinone ethyl *N*-(3:4-dioxocyclohexa-1:5-dienyl)aminoacetate (i.e. the ethyl ester of VII). The precise nature of *C* is less certain, but it would seem probable that it has been formed by condensation of two molecules of the quinone *B* to a tetrahydroxyphenazine derivative followed by a polymerization process. It is unlikely that it is derived from an indoxylquinone

or that it is closely related to the melanins which are brown in alkaline solution and have quite different absorption characteristics (Mason, 1948; Harley-Mason, 1948).

It is in any case clear that the further reactions undergone by such substances as *N*-(3:4-dihydroxyphenyl)aminoacetic acid are complex and may proceed either by formation of indole derivatives followed by oxidative polymerization, or by preliminary quinone formation followed by condensation reactions. In this sense the results obtained in the experiments described support the general thesis that the first step in the reaction of *o*-benzoquinone derivatives with amino acids or proteins involves the amino groups of the latter, yielding compounds in which nitrogen is directly attached to the aromatic nucleus in either the 4 or 5 positions or both. This step is followed by condensation and oxidation processes, yielding products which may bear some relation to those encountered in the melanin field but whose precise nature remains uncertain.

EXPERIMENTAL

Condensation of o-benzoquinones with aniline

Enzymic. To catechol (2 g.) in 0.1 M-sodium phosphate buffer (1 l., pH 6.7) aniline (5 ml.) and a trace of polyphenol oxidase (prepared according to Keilin & Mann (1938) from *Psalliota campestris*) were added and the solution aerated for 48 hr. with air washed with chloroform water. The solution rapidly turned red and the red precipitate which formed was collected by filtration, or preferably by centrifugation, washed with dilute HCl and with water and then dried *in vacuo* over P_2O_5 and KOH at room temperature. A further small quantity of material was obtained by extraction of the aqueous solution with ether. The dried material was extracted with acetone in a vapour-jacketed Soxhlet apparatus and the red needles which separated on concentration of the extract were crystallized from acetone to give 4:5-dianilino-1:2-benzoquinone (I) (2 g., 38%), m.p. 193°.

Similarly 3:4-dihydroxybenzoic acid (2 g.) gave 4:5-dianilino-1:2-benzoquinone (I) (1 g., 27%), m.p. 193°. (Found: N, 9.7. Calc. for $C_{18}H_{14}O_2N_2$: N, 9.7%.) The evolution of CO_2 in this reaction was demonstrated by using CO_2 -free buffer solution, CO_2 -free O_2 instead of air and passing the outgoing gases through a solution of $Ba(OH)_2$. During the reaction a copious precipitate of $BaCO_3$ formed. A control, in which buffer solution only was used, showed that no precipitate was formed in the absence of the other reactants.

3-Methylcatechol gave 4:5-dianilino-3-methyl-1:2-benzoquinone (III), red needles, m.p. 140°. (Found: C, 74.9; H, 5.2; N, 8.8. $C_{19}H_{16}O_2N_2$ requires C, 75.0; H, 5.3; N, 9.2%.)

4-Methylcatechol gave dianilino-4-methyl-1:2-benzoquinone (IV), red-brown needles, m.p. 202°. (Found: C, 78.8; H, 5.3; N, 10.8; active H, 0.49. $C_{20}H_{18}ON_2$ requires C, 79.1; H, 5.6; N, 11.1; active H, 0.53%.)

2-(3:4-Dihydroxyphenyl)propionic acid yielded red-brown needles of 2-(dianilino-1:2-benzoquinoneanil)propionanilide (V), m.p. 198°. (Found: C, 77.2; H, 5.4; N, 10.9. $C_{23}H_{20}O_2N_4$ requires C, 77.3; H, 5.5; N, 10.9%.)

Non-enzymic. Silver oxide (6.4 g.) (or silver acetate, 9.2 g.) was added to glacial acetic acid (150 ml.) and the suspension cooled to room temperature. A mixture of catechol (2 g.) and aniline (5 ml.) in glacial acetic acid (20 ml.) was added to the suspension of Ag_2O with shaking. A silver mirror formed and the solution became red. After being shaken for 1 hr., the solution was filtered, water (2 l.) added and the precipitate, collected by filtration and centrifugation, dried and purified by the method used in the enzymic experiment. Red needles of 4:5-dianilino-1:2-benzoquinone (3 g., 57%), m.p. 192°, were obtained.

Similarly 3:4-dihydroxybenzoic acid gave red-brown needles of 4:5-dianilino-1:2-benzoquinoneanil (II), m.p. 203°. (Found: C, 78.9; H, 5.5; N, 11.6. Calc. for $\text{C}_{22}\text{H}_{15}\text{ON}_3$: C, 78.9; H, 5.2; N, 11.5%). 3-Methylcatechol yielded 4:5-dianilino-3-methyl-1:2-benzoquinoneanil (VI), red-brown needles, m.p. 172°. (Found: C, 79.5; H, 5.6; N, 11.0. Calc. for $\text{C}_{25}\text{H}_{21}\text{ON}_3$: C, 79.1; H, 5.6; N, 11.1%). 4-Methylcatechol gave dianilino-4-methyl-1:2-benzoquinoneanil, m.p. 202° and 2-(3:4-dihydroxyphenyl)propionic acid gave 2-(dianilino-1:2-benzoquinoneanil)propionanilide, m.p. 198°.

Condensation of o-benzoquinone with amino acids and amines other than aniline

(a) Substitution of glycine (3.5 g.) for aniline in the above non-enzymic experiment gave, with catechol, 0.5 g. of a lustrous black solid, which was purified by concentrating filtered ethanolic solutions; m.p. >300°. (Found: C, 64.7; H, 4.4; N, 6.7. $\text{C}_{11}\text{H}_9\text{O}_3\text{N}$ requires C, 65.0; H, 4.5; N, 6.9%). Similar black solids were obtained from alanine, leucine, glycylglycine, phenylalanine and β -aminopropionic acid. Benzylamine or *N*-methylaniline gave red-brown solids, soluble in acetone and with m.p.'s >300°.

(b) To a vigorously stirred mixture of Ag_2O (4.75 g.), glycine (3.5 g.) and glacial acetic acid (150 ml.), a solution of catechol (2 g.) in glacial acetic acid (20 ml.) was added drop by drop during 30 min. A silver mirror formed and the solution became dark red. The solution was filtered, water (2.5 l.) added and the precipitate (1.8 g.) was collected, dried and purified as before; m.p. >300°. (Found: C, 69.5; H, 6.6; N, 2.7. $(\text{C}_{58}\text{H}_{68}\text{O}_3\text{N}_2)_n$ requires C, 69.7; H, 6.7; N, 2.8%).

Condensation of o-benzoquinones with insect cuticular protein

A solution containing water-soluble cuticular protein (50 mg.) from hawk moth larvae (*Sphinx ligustri*), 3:4-dihydroxybenzoic acid (100 mg.) and a trace of polyphenol oxidase in phosphate buffer (2 ml., pH 6.7) was set aside for 7 days and then filtered. Tetrabromophthalein ester and ninhydrin tests showed the absence of protein in the filtrate. A control, which contained only protein in buffer solution, gave strongly positive tests for proteins after 7 days. The substitution of catechol, 3:4-dihydroxyphenylacetic acid or 3:4-dihydroxyphenylalanine for the 3:4-dihydroxybenzoic acid also gave protein-free filtrates.

Synthetic experiments

4-Aminoveratrol. 4-Nitroveratrol (18.3 g.) in ethanol (250 ml.) was hydrogenated using a Raney nickel catalyst at room temperature and 100 atm. with stirring. The solution was filtered, the filtrate evaporated to dryness *in vacuo* and the solid distilled *in vacuo* to yield 15 g. (98%) of the base as colourless crystals, m.p. 87°. It gave a *hydrochloride*,

m.p. 246° (from ethanol). (Found: C, 50.3; H, 6.4; N, 7.6; Cl, 18.5. $\text{C}_9\text{H}_{12}\text{O}_4\text{NCl}$ requires C, 50.6; H, 6.5; N, 7.4; Cl, 18.7%.)

3:4-Dimethoxy-N-toluene-p-sulphonanilide. 4-Aminoveratrol (13 g.) and toluene-p-sulphonyl chloride (16.5 g.) were dissolved in dry pyridine (50 ml.), the solution set aside for 2 hr., the pyridine removed *in vacuo* and the residue dissolved in aqueous NaOH (10%, w/v). After extraction with ether, the aqueous layer was acidified to Congo red with HCl, the precipitate collected and recrystallized from ethanol (charcoal) to give the *anilide* (21 g., 81%) as colourless crystals, m.p. 141°. (Found: C, 58.3; H, 5.1; N, 4.6. $\text{C}_{16}\text{H}_{17}\text{O}_4\text{NS}$ requires: C, 58.6; H, 5.6; N, 4.6%). Treatment of the product with hot ethanolic KOH gave, on cooling, an 87% yield of the potassium salt.

N-(3:4-dimethoxyphenyl)-N-toluene-p-sulphonamidoacetic acid (IX). The above potassium salt (3.45 g.), chloroacetamide (0.94 g.) (or ethyl chloroacetate, 1.23 g.) and dry ethanol (20 ml.) were heated under reflux on a steam bath for 5 hr. After concentration *in vacuo*, aqueous NaOH (20 ml., 10%, w/v) was added and the solution boiled until evolution of NH_3 ceased (or, in the case of ethyl chloroacetate, boiled under reflux for 3 hr.). The resulting solution was acidified to Congo red, neutralized with solid NaHCO_3 and filtered, when 1.1 g. starting material was recovered. The filtrate was acidified to Congo red, giving a precipitate which was filtered off and recrystallized from water (charcoal) to give the *acid* (2 g., 55% or 85% on unrecovered starting material) as colourless needles, m.p. 157°. (Found: C, 55.5; H, 5.3; N, 3.9. $\text{C}_{17}\text{H}_{19}\text{O}_6\text{NS}$ requires C, 55.9; H, 5.2; N, 3.8%.)

Condensation of 3:4-dimethoxy-*N*-toluene-*p*-sulphonanilide with chloroacetic acid in aqueous ethanolic NaOH gave a lower yield (14%, or 74% on unrecovered starting material) of the same product.

N-(3:4-Dimethoxyphenyl)aminoacetic acid hydrochloride. 4-Aminoveratrol (7.65 g.), chloroacetic acid (2.36 g.) and water (12 ml.) were heated together on a steam bath for 5 hr. The solution was made alkaline, extracted with ether (3.2 g. of 4-aminoveratrol was recovered), the aqueous layer acidified to Congo red with HCl and again extracted with ether. The aqueous solution was evaporated to dryness under reduced pressure in an atmosphere of N_2 and the residue recrystallized from dry ethanol (charcoal) to give the amino acid hydrochloride (3.5 g., 57% on chloroacetic acid) as colourless plates, m.p. 182° (decomp.). (Found: C, 48.1; H, 5.7; N, 5.8. $\text{C}_{10}\text{H}_{14}\text{O}_4\text{NCl}$ requires: C, 48.5; H, 5.7; N, 5.7%.)

Alternatively, 4-aminoveratrol (7.65 g.), ethyl chloroacetate (3.06 g.) and ethanol (10 ml.) were heated together under reflux on a steam bath for 5 hr. Then KOH (3 g.) in methanol (90 ml.) was added and the solution heated under reflux for a further 2 hr. The solution was evaporated to dryness under reduced pressure, and in an inert atmosphere, the residue taken up in water, extracted with ether (3 g. 4-aminoveratrol recovered) and the aqueous solution rendered acid to Congo red. The *N*-(3:4-dimethoxyphenyl)-aminoacetic acid hydrochloride (4.9 g., 79% on ethyl chloroacetate; m.p. 182° decomp.) was isolated by the method given above.

4-Aminocatechol dibenzyl ether. 4-Nitrocatechol dibenzyl ether (25 g.; prepared by nitrating catechol dibenzyl ether) was heated on a steam bath with ethanol (200 ml.) and conc. HCl (5 ml.). Powdered Fe (reduced by H_2) (13 g.) was added in small portions and the solution boiled vigorously

under reflux for 3 hr. Then NaOH (2.5 g.) in ethanol was added and the solution filtered hot. The filtrate, on cooling, deposited colourless needles (20 g., 87%), m.p. 115°, unaltered on recrystallization from CHCl_3 (Balaban (1929) gives m.p. 92°). (Found: C, 78.6; H, 6.5; N, 4.7. Calc. for $\text{C}_{20}\text{H}_{19}\text{O}_2\text{N}$; C, 78.7; H, 6.3; N, 4.6%.) The hydrochloride crystallized from ethanol or water, containing HCl, as colourless needles, m.p. 206° (Balaban, 1929, gives m.p. 197°) and the hydrobromide had m.p. 195°. Acetylation of the free base with acetic anhydride in acetic acid gave 4-acetamidocatechol dibenzyl ether as colourless needles (from ethanol), m.p. 160° (Balaban, 1929, gives m.p. 150°). (Found: C, 75.9; H, 5.8; N, 4.3. Calc. for $\text{C}_{22}\text{H}_{21}\text{O}_3\text{N}$; C, 76.0; H, 6.1; N, 4.0%.)

Ethyl N-(3:4-dibenzoyloxyphenyl)aminoacetate. Ethyl bromoacetate (6.7 g.) was added to 4-aminocatechol dibenzyl ether (24.4 g.) in warm dry benzene (44 ml.) and the solution heated under reflux for 5 hr. on a steam bath. The cooled solution was extracted three times with warm ether (50 ml.) (the insoluble material (14.8 g.) was 4-aminocatechol dibenzyl ether hydrobromide, m.p. 195°), the ether evaporated and the residue crystallized from ethanol containing HBr to give colourless needles of the amino acid ester *hydrobromide* (14 g., 74%), m.p. 145°. (Found: C, 60.8; H, 5.6; N, 3.0; Br, 17.0. $\text{C}_{24}\text{H}_{26}\text{O}_4\text{NBr}$ requires C, 61.0; H, 5.6; N, 3.0; Br, 16.9%.) On grinding the hydrobromide with excess aqueous NaHCO_3 and crystallizing the residue from ethanol, *ethyl N-(3:4-dibenzoyloxyphenyl)aminoacetate* was obtained in theoretical yield as colourless crystals, m.p. 66°. (Found: C, 73.7; H, 6.3; N, 3.7. $\text{C}_{24}\text{H}_{26}\text{O}_4\text{N}$ requires C, 73.7; H, 6.4; N, 3.6%.)

N-(3:4-Dibenzoyloxyphenyl)aminoacetic acid hydrobromide monohydrate. *Ethyl N-(3:4-dibenzoyloxyphenyl)aminoacetic hydrobromide* (1.6 g.) and 2% methanolic NaOH (25 ml.) were heated together on a steam bath under reflux for 2 hr. Then HBr (sp.gr. 1.45; 3 ml.) was added and the solution evaporated to dryness under reduced pressure in an atmosphere of N_2 . Water (10 ml.) was added to the residue and the solution was filtered and evaporated; the dried residue on recrystallization from ethanol gave 1.5 g. (96%) of colourless crystals, m.p. 138°. (Found: C, 57.6; H, 5.0; N, 3.2. $\text{C}_{22}\text{H}_{24}\text{O}_6\text{NBr}$ requires C, 57.2; H, 5.3; N, 3.0%.)

Ethyl N-(3:4-dihydroxyphenyl)aminoacetate hydrobromide. *Ethyl N-(3:4-dibenzoyloxyphenyl)aminoacetate hydrobromide* (4.72 g.) in methanol (25 ml.) was hydrogenated using palladized charcoal (1 g. of 10%). The solution was filtered and evaporated to dryness under reduced pressure in an atmosphere of H_2 . The residual oil, on crystallization from a mixture of dry ethanol and ether, gave 2.6 g. (90%) of the *hydrobromide* as colourless needles, m.p. 166° (decomp.). (Found: C, 41.4; H, 4.7; N, 4.8. $\text{C}_{16}\text{H}_{14}\text{O}_4\text{NBr}$ requires C, 41.4; H, 4.8; N, 4.8%.) Acetylation with acetic anhydride in pyridine gave the *triacetyl* derivative of the base which formed colourless crystals m.p. 70°, when recrystallized from light petroleum (b.p. 100°–120°). (Found: C, 57.4; H, 5.6; N, 4.2. $\text{C}_{16}\text{H}_{13}\text{O}_7\text{N}$ requires C, 57.0; H, 5.7; N, 4.2%.)

Oxidation of ethyl N-(3:4-dihydroxyphenyl)aminoacetate hydrobromide. The amine hydrobromide (100 mg.) in 2 ml. of

methanolic formic acid (2 ml. 98% formic acid and 100 ml. methanol) was shaken for 1 min. with Ag_2O (200 mg.) and the solution filtered immediately. The deep-red filtrate *B* did not deposit any material when cooled to -70° , but after 24 hr. at room temperature a dark-violet pigment *C* separated. The observed molecular depression in the Rast method for determining molecular weights was of the order of one-tenth of that to be expected of a monomolecular oxidation product, indicating that the dark-violet pigment *C* was a compound of high molecular weight. The absorption spectra of these oxidation products were determined with a Beckman photoelectric quartz spectrophotometer, Model DU, using matched quartz cells of 1 cm. light path. The absorption maxima are recorded in Table 2.

Table 2. *Absorption maxima of oxidation products of ethyl N-(3:4-dihydroxyphenyl)aminoacetate*

Compound and solvent	Absorption maxima (μm)
Ethyl <i>N</i> -(3:4-dihydroxyphenyl)-aminoacetate hydrobromide (in dilute aqueous sulphurous acid)	295
4:5-Dianilino-1:2-benzoquinone (in methanol)	270, 790
3:5:6-Trihydroxyindole (in methanol)	292, 410
Yellow solution <i>A</i> with green fluorescence	260–290, 410
Red solution <i>B</i>	270, 397, 430
Dark-violet pigment <i>C</i> in water	275, 318, 552, 760

SUMMARY

1. The reaction of catechol derivatives with amines and amino acids in presence of chemical and enzymic oxidation systems has been studied.

2. Ethyl *N*-(3:4-dihydroxyphenyl)aminoacetate, believed to be the initial product of the reaction between *o*-benzoquinone and glycine ethyl ester, has been synthesized and its properties are described.

3. It is concluded that reaction between an *o*-benzoquinone derivative and an amino acid or protein, the first step in the hardening of the insect exocuticle, involves initially substitution at positions 4 and/or 5 of the quinone nucleus, the nitrogen of the amino groups becoming directly attached to the quinone nucleus; subsequently indole formation followed by oxidative polymerization may occur, or re-oxidation to the quinone form may be followed by complex condensation reactions.

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Studies of Sebum

4. THE ESTIMATION OF SQUALENE IN SEBUM AND SEBUM-LIKE MATERIALS

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Early in the present investigations it was found that squalene is a normal constituent of human sebum (MacKenna, Wheatley & Wormald, 1950); at about the same time Sobel (1949) showed that squalene is also a component of human ear wax, smegma and hair fat. In order to study the metabolism of this compound and its possible function in maintaining the health of the skin, it was necessary to devise a suitable method of estimation that could be applied to those small amounts of sebum that can be obtained from small, measured areas of skin.

Squalene is a rather inert substance and only two methods of estimation are available. First, it has a high iodine number (370) and an iodometric method of analysis was devised by Fitelson (1943) to estimate the squalene content of edible oils. Secondly, it gives a brown coloration in the Liebermann-Burchard test; this test has the disadvantage that colour develops slowly, though it can be made to develop more rapidly at slightly raised temperatures and if acetic anhydride is used as solvent instead of chloroform (Sobel, 1949). The hydrocarbon fraction of a lipid can be separated by chromatography on alumina after saponification and either the iodometric method or the colour test applied. These methods have been investigated and the conditions for best recovery established.

The previous paper in this series (Hodgson-Jones & Wheatley, 1952) dealt with the collection and estimation of sebum.

EXPERIMENTAL

*Methods for estimating pure squalene**Colorimetric method*

As described by Sobel (1949), this method consists of treating the sample of squalene (0.7 mg.) with 2 ml. of acetic anhydride and 5 drops of H_2SO_4 . The solution thus obtained is placed in a water bath at 38° for 30 min.; 5 ml. of CHCl_3 are then added. The resulting colour is an indefinite brown with greatest absorption at $400\text{ m}\mu$. but no well-defined maxima. It was observed that the colour did not completely develop in 30 min. and also that an increase in temperature hastened the rate of colour development; this effect was therefore studied in greater detail.

Effect of temperature on the rate of colour development. Samples of squalene (0.7 mg.) were treated with acetic anhydride (2 ml.) and H_2SO_4 (5 drops), then placed in a thermostat bath within the range 19 – 68° . At intervals duplicate samples were removed, 5 ml. CHCl_3 were added and the resulting colours read in the colorimeter (Evans Electroselenium portable model fitted with Ilford spectrum blue filter no. 621). Blank determinations were made with reagents incubated for the same length of time. Five series of determinations were performed at different temperatures and the results obtained (Fig. 1) show that the rate of colour development increases with increasing temperature, and even at the highest temperature studied does not appear to have reached a maximum in 30 min. At higher temperatures the blank reading becomes significant, and above 68° an intense colour develops rapidly in the blank, obscuring any colour produced by squalene in the test. The most convenient conditions for colour development appeared to be