the same equation was found to apply to data from experiments in which the equilibrium distributions of copper between soil and 0·33 M-Na_2SO_4 solution were determined for both a good and a bad soil under wide variations of experimental conditions (Fig. 3).

Data in Table 1 show, as might be expected, that the equilibrium concentration of copper in 0·33 M-Na_2SO_4 against a given concentration of copper on the *SOM* is independent of the solution volume.

DISCUSSION

In comparison with good Trinidad cacao soils, bad cacao soils are characterized by a low power to retain copper in complex with the *SOM*. Although the copper-retaining power of a soil is clearly related to its *SOM* content, the relation is not linear, and nearly all the bad soils lie on or near the steeply descending portion of the curve relating copper retention and percentage of *SOM* in the soil. Trace-element deficiencies, induced either by leaching or by a too great uptake of trace elements by past cacao crops, are, therefore, far more likely to occur in bad than in good soils.

Although difficulties in estimating small percentages of *SOM* in all probability caused the scatter of the points on the descending part of the copper-retention curve (Fig. 1), there is reason to believe that of the total value for percentage of *SOM*, as given by the method used, some 2·5 % represents absorptively inactive *SOM*. If it be assumed that some 2·5 % of the *SOM* is adsorptively inactive, two widely different soils give the same adsorption isotherm (Fig. 3), a fact that suggests there is no basic difference between the absorptive capacities of the organic fractions of the two soils.

It is reasonable to infer from Fig. 3 that the saturation capacity of *SOM* is about 1400 μ-equiv. Cu/g., to which value the logarithmically plotted Freundlich isotherm, known to be non-linear near saturation of the adsorbent, is asymptotic.

SUMMARY

1. Some bad Trinidad cacao soils have lower contents of organic matter, and lower copper-retaining powers than good soils.

2. The distribution of copper between a soil and a solution in equilibrium with it follows a Freundlich isotherm.

3. The significance of the results is discussed.

REFERENCES

Bremner, J. M., Mann, P. J. G., Heintze, S. G. & Lees, H. (1946). *Nature, Lond.*, **158**, 790.
Brun, T. S. (1945). *Bergens. Mus. Aarb.* **6**, 221.
Freundlich, H. (1930). *Kapillarchemi.* Leipzig: Acad. Verlags.
Hardy, F. (1929). *J. agric. Sci.* **19**, 727.
Hasler, A. (1943). *Mitt. Lebensm. Hyg., Bern.*, **34**, 79.
Lindow, C. W., Elvehjem, C. A. & Peterson, W. H. (1929). *J. biol. Chem.* **82**, 465.
McCreary, C. W. R., McDonald, J. A., Mulloon, V. I. & Hardy, F. (1943). *Trop. Agriculture, Trin.* **20**, 207.
Mellor, D. P. & Maley, L. (1948). *Nature, Lond.*, **161**, 436.
Sherman, G. D. & McHargue, J. S. (1942). *J. Ass. off. Agric. Chem., Wash.*, **25**, 510.

The Secondary Oxidation of Amino-acids by the Catechol Oxidase of Belladonna

By W. O. JAMES, E. A. H. ROBERTS, H. BEEVERS and P. C. DE KOCK
Oxford Medicinal Plants Scheme, Department of Botany, University of Oxford

(*Received* 13 *February* 1948)

The purpose of this investigation was to examine more fully than has hitherto been done the manner in which a plant polyphenolase is able to oxidize amino-acids in the presence of phenols. It was further hoped to discover which of the common amino-acids are liable to oxidation by the system, and the nature of the oxidation products. *Atropa belladonna* L. was known to possess an active polyphenolase system and was used because the nitrogen metabolism of this plant is under general investigation, and large quantities of reasonably homogeneous material were available.

EXPERIMENTAL

Preparation of the enzyme. Young leafy shoots of belladonna up to about 20 cm. long were harvested in May or June; 120–150 g. were disintegrated in a Waring Blendor with two successive portions of 350 ml. acetone. The acetone was filtered off, and the residue air-dried on a Buchner funnel and ground to a fine powder in a Wiley mill.

This crude powder had considerable polyphenolase activity; 50 mg. powder suspended in 2·5 ml. 0·01 M-catechol at pH 6 gave an O_2 uptake of over 200 μl. in 10 min. After three successive extractions with water at room temperature the powder still retained a high oxidase

activity, the loss varying somewhat with different batches of powder. The first washing showed oxidase activity equivalent to 1% of the activity of the unwashed powder. The third washing showed little or none.

The first washing gave a strongly positive ninhydrin reaction; but, after three extractions, further washings gave only a faint reaction. The powder therefore provided the desideratum of an active polyphenolase uncontaminated by free amino-acids. When dried by a further addition and removal of acetone it gave a stable preparation which kept its activity unimpaired for several weeks at least. In the following work the enzyme was invariably prepared in this way. Samples of the air-dry powder were weighed out as required.

Manometry. O_2 uptakes were measured in Warburg manometers using simple flasks with central chambers and single side arms (see Dixon, 1934, fig. 11*a*). CO_2 absorption was provided for, when required, by a roll of filter paper in the central chamber moistened with 20% KOH. In some experiments Keilin cups were used to provide a further possibility of mixing in the enclosed system. Most experiments were run in batches of five with a sixth manometer as thermobarometer. All experiments were of short duration, oxidation of the phenol usually being complete within 1 hr., and the secondary oxidation not being followed beyond a total period of 4 hr. It now seems that the results of earlier investigations of this system may have been vitiated by the dangerously long time interval, up to 40 hr., employed (e.g. Robinson & McCance, 1925).

Ammonia measurements. At the end of the manometric experiments, 0·5 ml. was withdrawn from the reaction mixture (total vol. 2·5 ml.). This was transferred to the outer circle of a standard Conway unit and the NH_3 content determined in the usual way, using boric acid as the absorbent and mixed bromocresol green and methyl red as indicator (Conway, 1947).

Isolation of the reaction products. It was anticipated that products of amino-acid oxidation might be formed according to the general scheme:

$$R.CH(NH_2).COOH + \tfrac{1}{2}O_2 \rightarrow R.CO.COOH + NH_3.$$

Attempts were, therefore, made to isolate carbonyl compounds as 2:4-dinitrophenylhydrazones. This was carried out in experiments starting with glycine and alanine. The details of the procedure differ so much in the two examples that they are described separately in the next section.

RESULTS

Oxidation of phenols in the absence of amino-acids

Oxidation of catechol. The progress of catechol oxidation by the belladonna enzyme is shown in Fig. 1. After 1 hr. 105 μl. of O_2 had been absorbed by 0·55 mg. catechol, corresponding to a mol. ratio O_2/catechol of approximately 1. Further O_2 uptake was slow.

Increasing the concentration of catechol from 0·001 to 0·01 M increased the rate of O_2 uptake (Fig. 2*B*). Raising the amount of enzyme present in 2·5 ml. from 12·5 to 100 mg. also accelerated O_2 uptake (Fig. 2*A*). Increased rates were, however, associated with earlier and more marked fallings away, so that the optimal conditions for catechol oxidation cannot be simply expressed. The nature of the 'inactivation' is examined further on p. 633. In the experiments on amino-acid oxidation, catechol was usually required to be present only in catalytic amounts. Concentrations of 0·005 or 0·002 M were commonly employed.

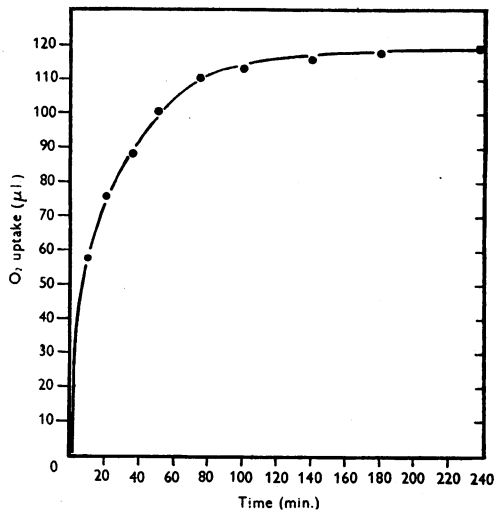


Fig. 1. Oxygen uptake over the first 3 hr. in the oxidation of 0·002 M-catechol by belladonna polyphenolase.

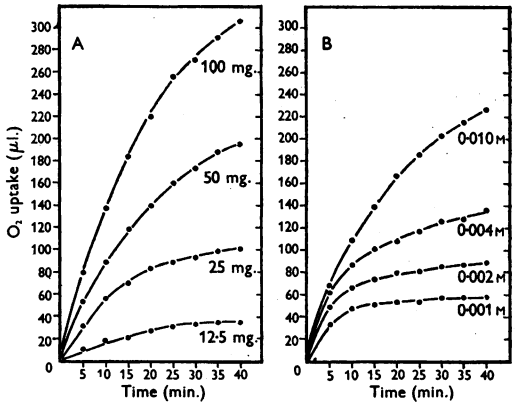


Fig. 2. *A*. Oxygen uptake during first 40 min. with 0·01 M-catechol in the presence of varying amounts of enzyme (in 2·5 ml. solution). *B*. Oxygen uptake with 50 mg. enzyme and varying concentrations of catechol.

The effect of acidity upon the rate of oxidation was examined over a pH range of 4·5–8·3, using phosphate buffers (Table 1). The effect of pH is clearly very slight over the range examined; autoxidation in the absence of enzyme was slow, and consumed only 8 μl. O_2 in 60 min. even at pH 7·8.

Table 1. *The effect of acidity on the oxidation of catechol by polyphenolase*

(Catechol, 0·005 M; 30°.)

pH	O_2 uptake (μl.) Time (min.) 5	15	30	45
4·5	42	97	128	145
5·6	49	105	133	150
6·6	50	100	129	149
7·7	53	106	140	159
8·3	51	107	141	160

changes of pH. The final O_2 uptake greatly exceeds 2 atoms O_2/mol. adrenaline and melanin-like pigments are formed (cf. Blaschko & Schlossmann, 1940).

Inhibitors. Since the enzyme is likely to have copper as its prosthetic group, the action of heavy-metal inhibitors was investigated over a range of concentrations. Cyanide (Table 4) was used at 0·01–0·001 M and sodium diethyldithiocarbamate ('dieca') (Table 5) at 0·005–0·0002 M. CO (Table 6) was tried at atmospheric pressure in a mixture of CO (approximately 80%) and O_2. Since no CO_2 production was to be expected, KOH was not put into the inner cups. There was not, therefore, any risk of loss of cyanide from the reaction mixture by distillation into alkali. The enzyme is markedly resistant to 0·001 M-cyanide. Plant polyphenolases have frequently been found to have a high resistance (Deb & Roberts, 1940; Roberts, 1941) and there seems to be much variation with species (Wieland & Sutter, 1930).

With diethyldithiocarbamate, inhibition is more pronounced than with cyanide, and is stronger in the weaker preparation in spite of the larger ratio of solid matter to dithiocarbamate. This is to be

Table 2. O_2 *consumption by belladona polyphenolase with various substrates*

Substrate	O_2 uptake (μl.)								
(Preparation A: 25 mg. in 2·5 ml. 0·066 M-phosphate, pH 7·1; 30°.)									
Time (min.) ...	10	20	30	40	60	70	90	120	150
0·008 M-*p*-Cresol	12	31	47	59	112	—	171	—	—
0·002 M-Catechol	65	94	100	—	113	—	124	—	—
0·008 M-Resorcinol	0	0	0	0	0	—	0	—	—
0·008 M-Hydroquinone	—	—	4	—	16	—	27	—	—
0·008 M-Phloroglucinol	45	90	127	—	—	—	—	—	—
0·008 M-Gallic acid	4	8	9	14	18	—	33	—	—
0·002 M-Adrenaline	19	44	59	74	—	—	—	—	—
(Preparation B: 50 mg. in 2·5 ml. 0·066 M-phosphate, pH 7·8; 30°.)									
0·002 M-*N*-Methyladrenaline	—	—	33	—	40	—	—	—	48
0·004 M-Aesculin	2	6	8	—	—	10	—	12	—
0·004 M-Aesculetin	25	39	48	—	—	62	—	69	—
0·002 M-Catechol	73	86	94	—	—	103	—	115	—

Oxidation of other phenols. Several other phenols were substituted for catechol as substrate (Table 2). Typical catechol experiments are included for comparison. The initial rate of oxidation is much faster with catechol than with any of the other phenols examined. Resorcinol is not oxidized and hydroquinone only very slowly, as would be expected. The increasing rate of *p*-cresol oxidation with time is also in accord with previous observations by other workers. The unexpectedly vigorous oxidation of phloroglucinol was confirmed in several further experiments; the O_2 consumption amounted to about 3 atoms/mol. phloroglucinol.

The oxidation of adrenaline (Table 3) was examined at different acidities and, unlike that of catechol, was found to be markedly sensitive to

Table 3. *The effect of acidity on the oxidation of adrenaline by polyphenolase*

(Adrenaline, 0·002 M; 30°.)

pH	O_2 uptake (μl.) Time (min.) 10	20	30	40	120	150
4·1	0	8	10	18	41	68
6·0	11	26	36	47	85	112
7·1	19	44	59	74	142	181
7·8	36	59	93	112	211	249

Table 4. *Inhibition of catechol oxidation by cyanide at* pH 6

(25 mg. polyphenolase; phosphate buffer; 2·5 ml. 0·005 M-catechol; 30°.)

Cyanide concn. (M)	O_2 uptake (μl.) Time (min.) 5	10	20	30	45
0·0	51	76	98	115	133
0·001	30	53	81	102	125
0·0013	17	31	48	67	89
0·002	10	14	21	27	40
0·01	0	0	0	0	0
% inhibition at 0·001 M	41	30	17	11	6

Table 5. *Inhibition of catechol oxidation by diethyldithiocarbamate*

(Preparation A, 25 mg.; preparation B, 50 mg.; each in 2·5 ml. 0·066 M-phosphate, pH 6; 2·5 ml. 0·005 M-catechol; 30.°)

Diethyldithiocarbamate concn. (M)	O_2 uptake (μl.) Time (min.) Preparation A 5	10	15	25	40	Preparation B 5	10	15	25	40
0·0	47	68	84	107	121	37	58	69	85	96
0·0002	41	60	73	96	115	25	41	51	69	84
0·001	25	38	51	68	85	13	23	30	41	51
0·002	—	—	—	—	—	11	23	30	39	48
0·005	15	26	32	45	51	2	12	17	23	25
% inhibition at 0·001 M	50	44	39	36	30	65	60	56	52	47

Table 6. *Inhibition of the polyphenolase activity of freshly expressed juice of belladonna by carbon monoxide*

(2 ml. juice; catechol 0·005 M; total vol. 2·5 ml.)

Conditions*		O_2 uptake (μl.) Time (min.) 5	10	15	20	25	30
Air		100	160	208	229	239	243
80% CO, 20% O_2	(*a*)	54	100	149	177	198	208
	(*b*)	60	115	151	179	206	215
	(*c*)	47	100	137	173	—	—

* Catechol tipped from side arm (*a*), 20 min., (*b*), 40 min., and (*c*), 60 min. after treatment with gas mixture.

Table 7. *Oxidation of ascorbic acid by polyphenolase*

(25 mg. polyphenolase in 2·5 ml. 0·002 M-catechol; phosphate buffer pH 6.)

Substrate	O_2 uptake (μl.) Time (min.) 5	10	20	30	45	60	90	125
0·002 M-Catechol	47	60	86	88	98	103	107	115
0·002 M-Catechol + 3·52 mg. ascorbic acid	48	86	123	139	169	191	214	237
3·52 mg. ascorbic acid	2	7	15	15	20	38	56	76

expected if the activity of the system depends upon the amount of copper present, since diethyldithiocarbamate is an almost specific copper precipitant.

In the experiments with CO, it was shown that the inhibitions could not be reversed by exposure to bright light. These results are in agreement with those of Kubowitz (1937) for potato polyphenolase.

Oxidation of ascorbic acid. The system is capable of acting as a continuous oxidation mechanism, and this was observed by providing ascorbic acid as substrate (Table 7). Glass-distilled water was used for all solutions. The slow continuous uptake of O_2 with ascorbic acid alone may indicate the presence of a direct ascorbic acid oxidase in the belladonna powder. The O_2 uptake with ascorbic acid and catechol present together exceeds the sum of the O_2 uptakes when they are present separately. It can, therefore, be concluded that the system catalyzes oxidation of ascorbic acid with catechol acting as redox body.

The oxidation of amino-acids and peptides in the presence of catechol

Oxygen uptake with catechol + glycine. Numerous experiments have been performed with mixtures of catechol and glycine. A typical set of results is in Table 8. The principal features evident in these results have been found in all experiments of the same kind. Glycine by itself is not oxidized, even slowly. Added to catechol it does not increase the rate of O_2 uptake very much during the first 20 or 30 min., by which time catechol oxidation is usually well advanced. During the second hour, the mixture of catechol and glycine continues to absorb O_2, and this still goes on in experiments of longer duration. It has been followed up to 4 hr. from the start (Fig. 3). The rate of amino-acid oxidation is always slow, relative to the initial rate of catechol oxidation, even under the most favourable conditions. Uptakes of 80–100 μl. O_2 are recorded in the first 10 min. with

Table 8. O_2 *uptake by polyphenolase with catechol and glycine*

(25 mg. polyphenolase in 25 ml. 0·066 M-phosphate pH 6; catechol, 0·002 M; glycine, 0·02 M; 30°.)

Substrate	O_2 uptake (μl.) Time (min.) 5	10	20	30	45	60	75	100	120
Glycine	0	0	0	0	0	0	0	0	0
Catechol	65	92	108	117	120	122	124	124	124
Glycine + catechol	56	84	104	116	125	135	142	148	157

catechol alone, but a period of about 3 hr. is necessary to raise O_2 consumption in the presence of glycine by the same amount, once catechol oxidation is complete. The time lag before an extra O_2 uptake

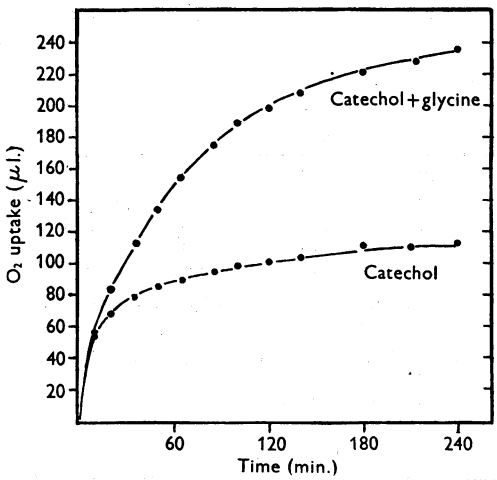


Fig. 3. Oxygen consumption over 4 hr. with 0·002 M-catechol (lower curve) and 0·002 M-catechol + 0·02 M-glycine (upper curve).

becomes evident is often more pronounced than in Fig. 3, and strongly suggests that the amino-acid is not being oxidized by a simple oxidation product of catechol, such as *o*-benzoquinone.

Ammonia formation with catechol + glycine. A series in which different flasks were opened and sampled after successive time intervals from 20 to 240 min. showed that NH_3 formation at pH 7·8 was progressive (Fig. 4). After 150 min. 198 μl. NH_3 had been produced. This is approximately twice the

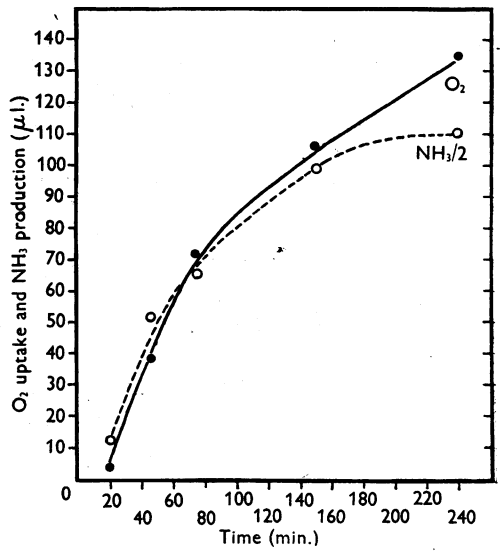


Fig. 4. Oxygen consumption and ammonia production with 0·002 M-catechol + 0·02 M-glycine. The ordinates represent μl. O_2 consumed and μl. ammonia/2 produced.

difference between the volume of O_2 consumed and that required for complete oxidation of the catechol present (227 − 130 = 97 μl. O_2). In other words, 1 mol. NH_3 had been released for each atom of extra O_2 consumed, as predicted by the equation on p. 627.

Table 9. O_2 *uptake and* NH_3 *production after incubation of polyphenolase with catechol and glycine at various* pH *values*

(25 mg. polyphenolase in 0·066 M-phosphate; catechol, 0·002 M; glycine, 0·02 M; 30°.)

pH	Time (min.)	O_2 uptake (μl.) Catechol only	Catechol + glycine	Extra uptake due to glycine	Ammonia production/2 (μl.)
4·2	210	127	134	7	6
6·0	120	124	157	33	33
7·1	150	133	250	117	100
7·1	180	115	255	140	116
7·8	225	120	326	206	130
7·8	240	130	264	134	110

After 240 min., the ratio NH_3/O_2 had fallen off, presumably owing to the entry of the NH_3 into side reactions. Other results of experiments lasting 2–4 hr. are shown in Table 9.

Absence of carbon dioxide production. Omission of KOH from the central cups produced no effect upon the pressure changes recorded at pH 7·1 with 0·002M-catechol and 0·02M-glycine. No CO_2 is released, therefore, but some might be retained in the buffer. When 0·5M-HCl was tipped into the reaction mixture after an uptake of 258 μl. O_2 and NH_3 production of 220 μl. in 3 hr., there was an output of 81 μl. CO_2; an output of 77 μl. CO_2 was obtained when acid was similarly tipped into a suspension of enzyme in buffer without added substrates. No CO_2 is produced, therefore, in the oxidation of catechol + glycine by our polyphenolase preparation.

The effect of acidity on the oxidation of glycine. Although the enzymic oxidation of catechol was found to be almost unaffected by changes of pH between 4·5 and 8·3, the secondary oxidation of glycine was markedly sensitive to changes in pH. This is in agreement with Oparin's (1927) observation that the rate of oxidation of amino-acids by chlorogenic acid increases between pH 7 and 12.

Formation of glyoxylic acid. Enzyme powder (250 mg.) was incubated with 5 ml. 0·01M-catechol, 12·5 ml. 0·1M-glycine and 12·5 ml. phosphate buffer (pH 7·8), and kept at 30° with occasional shaking. A red coloration developed rapidly, changing to dark purplish brown at the end of 3·5 hr. The solid enzyme was then filtered off; the purplish filtrate gave no reactions for proteins. On dilution of a small portion to a pale colour and addition of free tryptophan, a strong blue colour (Hopkins-Cole reaction) was given.

The remainder of the solution was treated with 0·5 vol. of 0·1% 2:4-dinitrophenylhydrazine in 2N-HCl, and allowed to stand in the refrigerator overnight. A dark granular solid which settled out was removed by decantation, and washed twice with 2N-HCl, twice with water and allowed to dry. After a short extraction with ethyl acetate, the bulk of the solid went rapidly into a yellow solution leaving a heavily coloured residue. The solution was decanted, and the ethyl acetate allowed to evaporate at room temperature. A yellow-orange crystalline product remained which gave a red colour with ethanolic NaOH and a very strong red colour with aqueous KOH. The crystals were long, feathery, branching plates, m.p. 200°. Mixed melting point with the reagent (2:4-dinitrophenylhydrazine) was depressed to 179°; mixed melting point with hydrazone freshly prepared from glyoxylic acid, 201°. Glyoxylic acid had, therefore, been isolated in identifiable quantities from the digest.

Oxidation of catechol and alanine. The oxidation of alanine was examined in the same way as that of glycine. At pH 6, O_2 uptake was not measurable over 4 hr. It was demonstrable at pH 7·1 and 7·8, but much slower than with glycine (Table 9). NH_3 production was correspondingly small, and the calculation of NH_3/O_2 ratios liable to too great an error to be of any value.

Formation of pyruvic acid. Only very small yields of the probable oxidation product, pyruvic acid, could be expected. In the light of previous experience (James & James, 1940) and after some preliminary trials, small yields were obtained by the following method. Enzyme extract (10 ml.) was incubated at 30° with 0·005M-catechol; 9 mg. alanine, an excess of $CaCO_3$ and toluene were added and the vol. made up to 20 ml. After 16 hr. the digest was distilled under reduced pressure. The distillate was discarded and the digest acidified with dilute H_2SO_4, filtered and again distilled *in vacuo*. About 20 ml. distillate was collected, and 10 ml. 0·2% 2:4-dinitrophenylhydrazine added (less would have been better). After standing, the yellow solution was extracted with ethyl acetate. The ethyl acetate solution was extracted twice with half-saturated Na_2HPO_4 solution. The aqueous layer was separated and acidified with 2N-HCl and then extracted with ethyl acetate. A further transfer was made into 0·066M-Na_2HPO_4 which took only a small fraction of the coloured material from the ethyl acetate. The dilute alkali fraction was separated, acidified and returned to ethyl acetate. This was allowed to evaporate. A few yellow crystals were obtained. After further solution in dilute sodium phosphate, and recrystallization from ethyl acetate, crystals were obtained as diamond-shaped bright yellow platelets with m.p. 215°. Melting point of freshly prepared hydrazone of redistilled pyruvic acid, 216°.

Oxidation of other amino-acids and dipeptides. The other amino-acids are oxidized much more slowly than glycine, and the alanyl dipeptide more slowly than the glycyl dipeptides (Table 10). For each atom of O_2 consumed in the oxidation of glycine there is rather less than 1 mol. of NH_3 released, probably due to the utilization of NH_3 in some side reaction. With the much slower rates of oxidation of the other amino-acids the discrepancy becomes much larger.

Absence of an amino-acid dehydrogenase from the insoluble enzyme preparation

The observation that the secondary oxidation is highly sensitive to changes of pH might suggest that it was catalyzed by an amino-acid dehydrogenase. The possibility can, however, be discounted for the following reasons: (i) The conditions of preparation of the enzyme are such that no dehydrogenase could be expected to survive. (ii) Malachite green, a reputed dehydrogenase poison (Boswell & Whiting, 1938), was found to inhibit the oxidation of catechol alone and catechol + glycine only slightly at a concentration of 0·002M. (iii) The polyphenolase is unusually resistant to heat for an enzyme, and heating at 100° for 5 min. diminished the oxidation of catechol + glycine as well as of catechol alone only to a very slight extent. (iv) The oxidation can be reproduced, including NH_3 production, if O_2 and polyphenolase are replaced by $K_3Fe(CN)_6$, and no enzyme is present in the system at all. (v) Secondary amines are not oxidized, but do form a coloured complex (Beevers & James, 1948), and there is no diminution in the rate of amino-acid oxidation if the enzyme is filtered off after forming the coloured complex with a secondary amine. On addition of amino-acid, a normal rate of oxidation is catalyzed by the coloured complex. Since the enzyme is exhaustively extracted beforehand with water, none can be supposed to remain behind in solution. From these facts it seems clear that no enzyme was catalyzing the secondary oxidation of the amino-acid in our extracted system; but this does not necessarily rule out the existence and participation of such an enzyme in the intact cell.

Table 10. O_2 *uptake and* NH_3 *production after incubation of polyphenolase with catechol and various amino-acids*

(25 mg. polyphenolase, 0·002 M-catechol + 0·02 M-amino-acid; phosphate buffer pH 7·8; vol. 2·5 ml.; 30°.)

Amino-acid	O_2 uptake (μl.) Catechol only	Catechol + amino-acid	Difference	Ammonia production/2 (μl.)
Glycine	130	264	134	110
DL-Alanine	120	150	30	9
DL-Phenylalanine	130	169	39	21
DL-Aminobutyric acid	130	154	24	12
L(?)-Methionine	130	126	—	16
L-Valine	130	103	—	7
L-Leucine	130	191	61	37
DL-Isoleucine	130	174	44	13
L(?)-Aspartic acid	130	153	23	7
L-Glutamic acid (163 min.)	133	179	46	18
L-Histidine	130	152	22	9
L(?)-Tryptophan	130	163	33	13
Glycylglycine	130	211	81	45
Glycyl-L(?)-leucine	130	192	62	43
DL(?)-Alanylglycine	130	133	3	8

Formation and activity of a coloured complex

When catechol alone is oxidized by the enzyme a pale yellow colour is produced which slowly turns brown. With the low concentrations of catechol employed in these experiments the colour was rarely deeper than pale straw, even after 4 hr. Addition of amino-acid caused the rapid formation of a characteristic intense red irrespective of the rate at which the particular amino-acid was oxidized. Alanine produced a full development of the colour at pH 6, although its O_2 uptake was negligible at this pH and much less than that of glycine at pH 7·8 (Table 10).

A similar reaction is given with amino-acids present in belladonna extracts from resting tissues. On addition of catechol to a suspension of the unwashed acetone preparation of the enzyme, the typical red colour develops. The enzyme, which has been washed with water until the washings are no longer capable of giving a ninhydrin reaction, fails to form the colour when incubated. For this reason the washed insoluble enzyme was particularly convenient material for the present investigation. It was observed that colour formation is much more rapid at pH 7·8 than at pH 6. This effect could also be reproduced if the enzyme and O_2 were replaced by inorganic oxidizers. Treatment with ceric sulphate in acetate buffer at pH 5 oxidizes catechol immediately (Ball & Chen, 1933), but we found that colour formation with glycine or proline was slow, extending over 15 min. Oxidation at pH 7·8 with potassium ferricyanide in phosphate buffer led to colour formation almost immediately. This observation is significant in the light of the conclusion of Nelson & Dawson (1944) that conversion of *o*-benzoquinone to *p*-hydroxy-*o*-quinone is relatively slow below pH 7.

Accurate correlation of the amount of colour produced with the amount of oxidation performed is not very easy if enzyme and O_2 are the agents. It is more readily carried out using potassium ferricyanide as oxidizer. Two mol. of ferricyanide are equivalent to 1 atom of O_2 and oxidize 1 mol. catechol to *o*-quinone. It has been shown on p. 627 that the complete oxidation of the catechol molecule requires 2 atoms O_2 ($\equiv 4K_3Fe(CN)_6$). By means of measurement in a Spekker absorptiometer it was found that $4K_3Fe(CN)_6$ is also needed for the development of the full colour from 1 mol. catechol in the presence of an excess of glycine.

Colour formation is reversible. The colour is bleached by ascorbic acid or by magnesium + dilute acetic acid and restored by shaking in air. It is also bleached by reduction with bisulphite. Separation of coloured and leuco forms of the compound has been obtained by shaking a deeply coloured solution with charcoal for a few minutes at room temperature, and then filtering. The filtrate is completely colourless, but on standing in air becomes coloured again. The amount of colour formed by the second oxidation is small compared with the original. In this way, the coloured compound may be produced slowly from the leuco form, even in the absence of enzyme, at a stage when catechol is no longer present, and it may be bleached with hydrogen donors. The leuco and coloured compounds are likely, therefore, to resemble phenolic and quinonoid forms respectively, and may be capable of transferring hydrogen reversibly.

The coloured complex, as produced by the action of potassium ferricyanide on catechol and glycine,

is able to effect oxidation of glycine in the absence of any enzyme. With ferricyanide, catechol and glycine present in the molecular proportions of 4 : 1 : 10 (0·55 mg. catechol in 2·5 ml. phosphate buffer pH 7·8) an uptake of O_2 and production of NH_3 were clearly demonstrable (Table 11). Both these were

Competition between catechol and reduced coloured complex. After a period of about 60 min., oxidation of catechol becomes very slow. This is not due to inactivation of the enzyme, since on addition of fresh catechol the rate of O_2 uptake again becomes rapid (Fig. 5, curve I). If glycine is present with

Table 11. O_2 *uptake and* NH_3 *production under varying conditions of oxidation*

(Wts. of reagents, *a*, *b*, *c*, *d* in mg.; phosphate buffer pH 7·8; total vol. of reaction mixture, 2·5 ml.)

	Wt. of reagents (mg.)				Molecular ratio	O_2 uptake (μl.) Time (min.)									Ammonia production (μl.)
Exp.	Enzyme (*a*)	$K_3Fe(CN)_6$ (*b*)	Catechol (*c*)	Glycine (*d*)	*b* : *c* : *a*	10	20	30	45	60	75	155	180	240	240
1	—	6·6	0·55	3·75	4 : 1 : 10	—	10	36	61	88	110	197	220	262	54
2	25	6·6	0·55	3·75	4 : 1 : 10	11	24	50	81	111	140	264	288	314	87
3	25	6·6	1·10	—	4 : 2 : 0	48	63	74	78	84	89	112	118	137	10
4	25	—	0·55	—	0 : 1 : 0	48	63	73	78	90	95	113	113	123	8
						Control, reagents only at zero time									9

higher in the presence of enzyme, indicating that the secondary oxidation is indirectly accelerated by the enzyme, though autoxidation without it is quite marked. The experiment shows unequivocally that the coloured complex can bring about the breakdown of glycine directly without the action of any enzyme

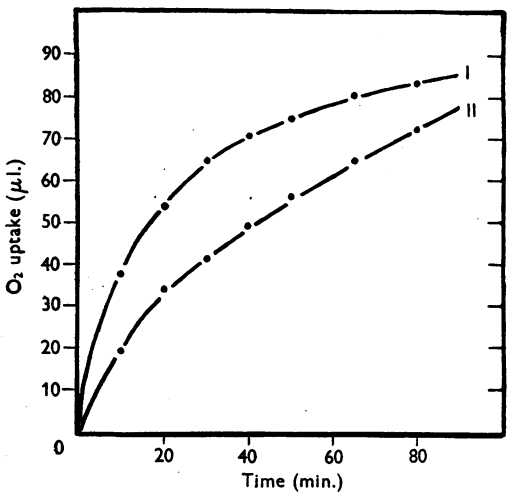


Fig. 5. Oxygen consumption after an initial period of 60 min. which is not included in the graph. Curve I: 0·002 M-catechol present at start and a second equal amount added at 60 min.; curve II: 0·002 M-catechol +0·02 M-glycine present at start, an equal amount of catechol added after 60 min.

(Table 11, line 1). The increased breakdown of glycine in the presence of the polyphenolase (line 2) is due to faster formation and regeneration of the coloured complex, and it has already been shown (p. 631) that no second enzyme intervening between the coloured complex and the amino-acid is likely to be present in our preparations.

catechol from the beginning of the experiment, a moderate rate of O_2 uptake continues steadily over a long period. The addition of extra catechol to the mixture after 60 min. causes an increase in the rate (Fig. 5, curve II), which is not as great as the increase obtained in the absence of the amino-acid. It cannot, therefore, be supposed that the presence of the amino-acid tends to retain the catechol in the oxidizable form, as would occur if it were itself being oxidized by the initial catechol oxidation product (*o*-quinone). A more probable explanation of these results is that the reduced form of the coloured complex competes with catechol for the enzyme, and is itself oxidized, but more slowly than catechol.

Relation of amino-acid oxidation to amino-acid concentration. The extent of amino-acid oxidation can be estimated either by the NH_3 produced, or by the O_2 consumed over and above that required for oxidation of the catechol. Data are available from a series of experiments in which NH_3 and O_2 changes were recorded with varying amounts of amino-acid. In all of them 25 mg. of the same enzyme preparation were used with 0·002 M-catechol in 2·5 ml. phosphate buffer at pH 7·8. The experiments were carried on in the manometers for 4 hr. The results are expressed in Figs. 6 and 7 by plotting μl. NH_3 and O_2 against the mol. ratio glycine/catechol. Both for NH_3 and O_2 the relation is linear from 1 to 10 mol. glycine/mol. catechol. In neither graph does the line spring from the origin. Some NH_3 is produced from the enzyme in the absence of added glycine, and no increase is recorded with additions of 0·5 and 1·0 mol./mol. catechol. Values < 1 mol. are clearly off the line and their departure is statistically significant. The mean deviation of three experiments at zero concentration of glycine from the regression line of NH_3 production in glycine concentration is 22·4, with a calculated probability of being on the line of only 0·001. The results of this experiment indicate that there is no

oxidation of amino-acid until there is an excess of glycine over 1 mol. for each mol. of catechol; or, in other words, that 1 mol. of amino-acid enters into combination with the catechol oxidation products before oxidation of amino-acids can occur.

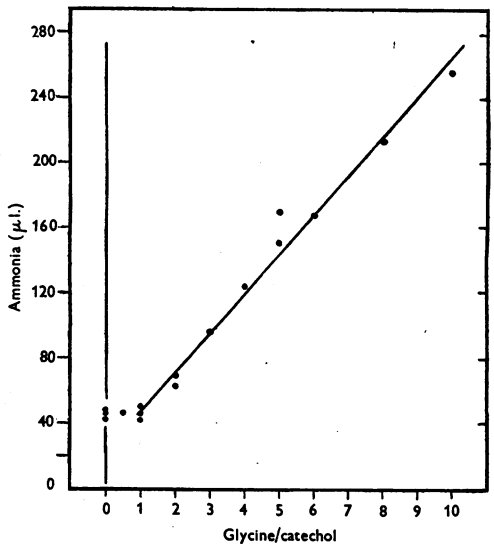


Fig. 6. Ammonia production with varying molar concentrations of glycine. Catechol concentration 0·002M throughout.

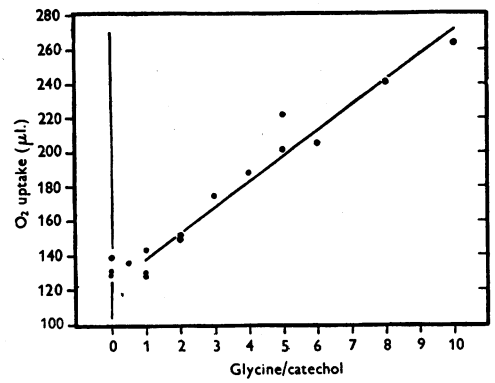


Fig. 7. Oxygen consumption with varying mol. ratios of glycine and catechol; the catechol concentration was 0·002M.

The data for O_2 consumption are consistent with this idea, though they are not in themselves so conclusive, on account of the greater error involved in allowing for the O_2 consumed in the primary catechol oxidation. Reference to Fig. 7 shows that the results for 0 and 0·5 mol. glycine/mol. catechol again appear to be off the regression line. There are three results available for zero concentration with a mean deviation of 10·3 from the value predicted by the regression. Calculation by the usual methods shows a probability of its belonging to the curve of only 0·07 (about 1 in 14·5). There is no evidence of additional O_2 consumption until 1 mol. of glycine has been fixed for every molecule of catechol originally present.

Substitution of adrenaline for catechol leads to an interesting result. The effect on the O_2 uptake of adding glycine to the adrenaline is masked by the complexities of the further oxidations of adrenaline itself (p. 628). NH_3 is not released in these adrenaline reactions, but is formed when glycine is added. The effect of varying the concentration of glycine is shown in Fig. 8, and it is clear that the curve proceeds linearly to the zero value. In other words, the

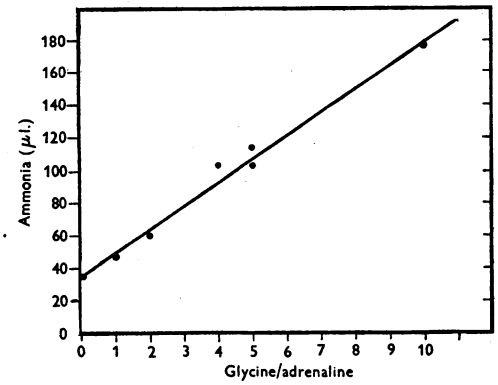


Fig. 8. Ammonia production from glycine by polyphenolases and adrenaline. Adrenaline concentration 0·002M; mol. ratios of glycine/adrenaline varying from 0 to 10.

amino-acid is oxidized without the prior formation of a coloured complex involving the amino-acid. The place of the coloured complex is taken by the adrenochrome produced by the oxidation of adrenaline (Blaschko & Schlossmann, 1940), and the combination of 1 mol. of amino-acid with the intermediate oxidation product is no longer necessary before oxidation of glycine can take place.

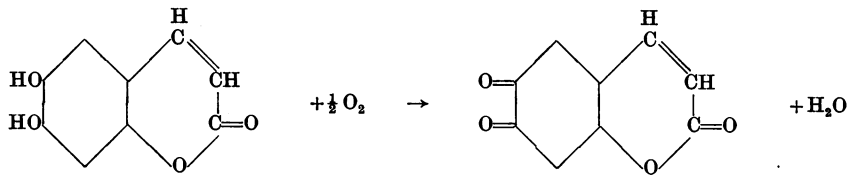
N-Methyladrenaline and aesculetin are also oxidized by the polyphenolase at readily measurable rates (Table 2), but they do not form a coloured compound like adrenochrome either alone or with addition of amino-acid. They are also unable to set up an oxidation system for glycine.

DISCUSSION

The secondary oxidation of amino-acids by polyphenolase systems has been assigned considerable importance in the nitrogen metabolism of plants, but has not been examined extensively. The general characteristics of the system, as it exists in *Atropa belladonna*, are shown by the experiments described above. The enzyme oxidizing the polyphenol can be prepared in a highly active, insoluble form by

precipitation with acetone. From this the remaining polyphenols and amino-acids can be washed out with water, leaving a large proportion of the original polyphenolase activity in the precipitate. Pure catechol is vigorously oxidized by this precipitate with the uptake of approximately 2 atoms O_2/mol. catechol oxidized; and the greater part of the enzymic activity remains unimpaired when this has been done.

In the absence of other oxidizable substances, brown condensation products of the catechol appear. The oxidation of catechol is little affected by changes of pH over the range 4·5–8·3; but other polyphenols

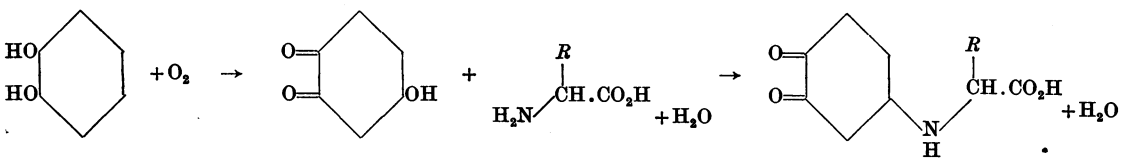


may behave differently, since the oxidation of adrenaline was found to be much faster at pH 7·8 than at 4·1, and the total uptake of O_2 much greater than with catechol. The enzyme is poisoned by 0·001 M-diethyldithiocarbamate and, therefore, probably contains copper; but, like many other plant polyphenolases, it is markedly resistant to 0·001 M-cyanide. Catechol is reduced again by ascorbic acid, for example, and a continuous oxidation system with catechol as carrier can be set up. It would be natural to suppose at first that the progressive oxidation of amino-acids was similarly achieved, but the results obtained suggest a rather more complex mechanism. On addition of amino-acid to the enzyme-catechol mixture a rich red coloration develops, altogether different from the brown colours with catechol alone. Similar colours are given by some other primary amines. The addition of one equivalent of amino-acid does not result in the release of any ammonia, and with further additions of amino-acid there is a linear increase in the amount of ammonia released.

is also faster at pH 7·8 than in slightly acid solutions, whether inorganic oxidizers or enzyme + O_2 were used. It appears probable, therefore, that the coloured compound is formed by the reaction of a molecule of *p*-hydroxy-*o*-quinone with a molecule of amino-acid. This amino-acid molecule is not subsequently released in oxidized form. Its condensation with the quinone precedes the oxidation of further amino-acid.

Condensation of the amino-group at the 4 position of the nucleus is confirmed by the inability of aesculetin, in which this position is already occupied, to form a coloured complex, even when oxidized (p. 634). The O_2 uptake in this case was equivalent to less than 1 atom of O_2/mol. of aesculetin. It would, therefore, appear that no hydroxyl group is attached and no condensation occurs in positions adjacent to the *o*-hydroxyl groups. Further confirmation is afforded by the behaviour of adrenaline. On oxidation it forms adrenochrome (Green & Richter, 1937; Blaschko & Schlossmann, 1940), which is not unlike our coloured compound in tint and intensity of colour. Ring closure occurs at the 4 position; no ammonia is released, but the adrenochrome formed is able to oxidize glycine with liberation of ammonia from the amino-acid. With the fully *N*-substituted *N*-methyladrenaline which is a tertiary base, no such cyclization can occur and the oxidation of glycine is not catalyzed.

Assuming, for the above reasons, that the coloured compound is formed by condensation of amino N at position 4 on the nucleus, we may write its formation as follows:



The oxidation of 1 mol. of catechol to *o*-benzoquinone requires only a single atom of O_2 or its equivalent. Reasons have been advanced by Nelson & Dawson (1944), using potato tyrosinase, for supposing that the consumption of the second O_2 atom leads to the formation of *p*-hydroxy-*o*-quinone, which occurs slowly at pH 4 and faster at pH 7. It is significant that colour formation in our experiments

The coloured condensation product, which seems at present to be best formulated as a substituted *p*-amino-*o*-quinone, does not condense rapidly like *p*-hydroxy-*o*-quinone, but becomes the actual oxidizer of the amino-acid. The participation of the enzyme may be limited to its initial formation. Once the coloured compound is formed, its reduction by the amino-acid is non-enzymic, and its reoxidation in

the presence of oxygen may occur spontaneously also. Nevertheless, the reduced coloured compound is capable of uniting with the enzyme surface (p. 633), where its reoxidation appears to be accelerated.

SUMMARY

1. From *Atropa belladonna* leaves a catechol oxidase has been isolated which is highly active in an insoluble form. It was obtained free from polyphenols and amino-acids.

2. It was found to oxidize catechol with the rapid uptake of approximately 2 atoms of oxygen/mol. catechol; further oxygen uptake was slow.

3. Phloroglucinol, *p*-cresol, adrenaline, *N*-methyladrenaline and aesculetin were also oxidized rapidly; hydroquinone and gallic acid more slowly; aesculin very slowly and resorcinol not at all.

4. Variation of pH between 4·5 and 8·3 had little effect on the rate of oxidation, cyanide inhibited very slightly; but diethyldithiocarbamate more strongly. Carbon monoxide inhibited, and the inhibition was not reversible by light.

5. Secondary oxidation of glycine took place with release of ammonia and uptake of additional oxygen. The volume of ammonia produced was approximately double the volume of extra oxygen consumed. Glyoxylic acid was isolated from the reaction products. No carbon dioxide was released. Pyruvic acid was similarly obtained from alanine, but the reaction was much slower than that with glycine.

6. Other amino-acids and dipeptides were oxidized much more slowly than glycine, and some not at all.

7. Formation of a red colour preceded the secondary oxidation. Once it is formed, its reduction by the amino-acid is non-enzymic, and its reoxidation in the presence of oxygen may occur spontaneously. Nevertheless, the reduced coloured complex is capable of uniting with the enzyme surface, and its reoxidation may be accelerated. Reasons are given for supposing that the coloured complex, probably a *p*-amino-*o*-quinone, is the immediate oxidizer of the amino-acid, and that the role of the polyphenolase is limited to its formation and possibly also its regeneration in the system.

The authors had the pleasure and profit of frequent discussion with Dr E. M. Trautner while this work was in progress. They are indebted to Dr H. Blaschko for a sample of *N*-methyladrenaline.

REFERENCES

Ball, E. G. & Chen, T. T. (1933). *J. biol. Chem.* **102**, 691.
Beevers, H. & James, W. O. (1948). *Biochem. J.* **43**, 636.
Blaschko, H. & Schlossmann, H. (1940). *J. Physiol.* **98**, 130.
Boswell, J. G. & Whiting, G. C. (1938). *Ann. Bot., Lond.*, **2** (New Series), 847.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*. London: Lockwood.
Deb, S. B. & Roberts, E. A. H. (1940). *Biochem. J.* **34**, 1507.
Dixon, M. (1934). *Manometric Methods*. Cambridge: University Press.
Green, D. E. & Richter, D. (1937). *Biochem. J.* **31**, 596.
James, G. M. & James, W. O. (1940). *New Phytol.* **39**, 266.
Kubowitz, F. (1937). *Biochem. Z.* **292**, 221.
Nelson, J. M. & Dawson, C. R. (1944). *Advanc. Enzymol.* **4**, 99.
Oparin, A. (1927). *Biochem. Z.* **182**, 155.
Roberts, E. A. H. (1941). *Biochem. J.* **35**, 1209.
Robinson, M. E. & McCance, R. A. (1925). *Biochem. J.* **19**, 251.
Wieland, H. & Sutter, H. (1930). *Ber. dtsch. chem. Ges.* **63**, 66.

The Behaviour of Secondary and Tertiary Amines in the Presence of Catechol and Belladonna Catechol Oxidase

By H. BEEVERS and W. O. JAMES
Oxford Medicinal Plants Scheme, Department of Botany, University of Oxford

(*Received* 13 *February* 1948)

The results presented in the preceding paper (James, Roberts, Beevers & de Kock, 1948) showed that a polyphenol oxidase from belladonna, using catechol as an intermediate, was capable of oxidizing certain primary amino-acids. The present paper describes the effects of the same enzyme system on secondary and tertiary amines, and, in addition, the oxidation of glycine which occurs when it is added to a complex of catechol and secondary amine in the presence of the enzyme.

EXPERIMENTAL

The enzyme was prepared as described in the preceding paper (James *et al.* 1948) and the manometric technique was essentially the same; the experiments were carried out at 30°, and the solutions were buffered to pH 7·8 by the addition of 0·1 M-phosphate mixtures. When two reactants were to be added separately to the main mixture in the manometer vessels, Keilin cups were employed.