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Incorporation of Glycine into Glutathione and Fibrinogen of Rats under Adrenaline Treatment

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Subcutaneous injections of adrenaline cause in the rat an increase in the concentration of plasma fibrinogen (Henriques, Henriques & Mattos, 1950). This effect seems to be specific for adrenaline, since other sympathomimetic amines and their derivatives, administered in the same dose, cause a much lower increase in fibrinogen concentration, or have no effect at all (Henriques, Henriques & Levy, 1956). In the present work an attempt has been made to elucidate whether the adrenaline-induced increase in fibrinogen is produced by a simple liberation of the protein from its possible stores or is caused by a more direct action of adrenaline on the metabolism of fibrinogen by stimulating its synthesis. For this purpose the specific radioactivity of glycine isolated from fibrinogen of rats treated with adrenaline was compared with that of controls injected with an equal volume of saline at various times after administration of [α - 14 C]glycine, the concentration of fibrinogen being determined at the same time. To control the specificity of the action of adrenaline on fibrinogen, the incorporation of labelled glycine into the mixed serum proteins was determined simultaneously. As there is the possibility that liver glutathione plays some role in protein synthesis, the incorporation of labelled glycine into this tripeptide under adrenaline treatment was also studied. In connexion with this the concentration of glutathione in the liver of rats was also determined at different times after adrenaline injection.

METHODS

General. Female albino rats (96), weighing 150–180 g., were divided at random into 12 groups of 8 rats each, and used in the incorporation experiments. Half of these rats received subcutaneously 1 μ g. of adrenaline/g. of body wt. in 0.1–0.3 ml. of solution, and the other half received the same volume of saline. All the animals were fasted after these injections, which were done alternately, one rat receiving

adrenaline and the other saline 5 min. later, in order to reduce the differences caused by the conditions of the animals and to allow accurate timing of the subsequent glycine injection and killing. Two hours after the adrenaline or the saline treatment each rat was injected intravenously, through a tail vein, with 2.5 μ C of [α - 14 C]glycine (0.12 mg. dissolved in 0.125 ml. of 0.85% NaCl)/100 g. of body wt. Exactly 10 min., 20 min. and 1, 2, 3 and 4 hr. respectively after glycine injection, the animals were bled from the aorta under anaesthesia (pentobarbitone sodium, 4 mg./100 g. of body wt.) and their livers were quickly removed, sliced, pressed against blotting paper and frozen in dry ice. Equal weights (usually 5 g.) of liver from each rat from the same group were pooled, finely pulverized in a mortar while frozen by dry ice, and immediately extracted with a solution of trichloroacetic acid (TCA) in the manner subsequently described. The blood was collected with a syringe coated with silicone, Dry Film 9987 (General Electric Co., Pittsfield, Mass., U.S.A.), and clotting was prevented by the addition of 0.01 ml. of 30% (w/v) potassium oxalate/ml. of blood.

Twenty female albino rats, weighing 210–225 g., were divided at random into 4 groups of 5 rats each, and used for investigating the influence of adrenaline on the concentration of liver glutathione (GSH). The animals of group 1 received no treatment and served as controls, whereas those of groups 2–4 received subcutaneously 1 μ g. of adrenaline/100 g. of body wt. At 60, 150 and 250 min. after adrenaline injection the animals of groups 2–4 respectively were killed, under anaesthesia as previously described, by section of the inferior vena cava between the liver and the heart, the liver being removed after pressing out most of its blood. The controls were killed in the same way. A portion (1 g.) of the liver was extracted in a Potter homogenizer for a few minutes with 4 vol. of 0.14N-sulphosalicylic acid. The extract obtained was centrifuged and the clear supernatant was kept frozen until the determination of GSH, which took place at most 24 hr. later.

Estimation and isolation of fibrinogen. Plasma (0.5 ml.) from each rat was used for the determination of fibrinogen by the technique of Cullen & Van Slyke (1920) with ashing by the method of Campbell & Hanna (1937), distillation and titration of ammonia. The rest of the plasma was used for the isolation of fibrinogen as fibrin, also by the technique of

Cullen & Van Slyke (1920), after mixing equal volumes of plasma of each rat from the same group. The fibrin clot was pressed against filter paper, suspended in 10 ml. of 0.9% NaCl containing 2 mg. of inactive glycine and left in the cold for 24 hr. with occasional stirring. With the same technique the fibrin was further washed four times with 0.9% NaCl.

Extraction of free amino acids and glutathione. The diluted serum obtained after the separation of fibrin was freeze-dried and the dry residue dissolved in a small volume of water. This solution or the liver powder was treated with an equal weight of 10% (w/v) TCA. After being thoroughly mixed and left standing for at least 10 min., the mixtures were centrifuged. The precipitated proteins were resuspended in half the initial volume of 5% (w/v) TCA and this extraction was repeated twice. An amount equivalent to about one-third of the pooled livers and the whole-serum extract was used for glycine isolation, and the remaining two-thirds of the pooled livers were used for the isolation of GSH.

Isolation of glutathione. The glutathione was precipitated as cuprous mercaptide (GSCu) by the method of Hopkins (1929), with preliminary separation as cadmium mercaptide (Waelisch & Rittenberg, 1941).

Separation of serum proteins. The washed serum proteins were resuspended in 10 ml. of 5% (w/v) TCA containing inactive glycine (20 mg./100 ml.) and left at room temperature for 24 hr. with occasional stirring. The proteins were then separated by centrifuging, resuspended in 5% (w/v) TCA, left for 24 hr. with occasional stirring and centrifuged again. This treatment was repeated four times. The proteins were further washed three times with acetone, extracted once with a boiling ethanol-ether mixture (3:1, v/v) and dried over P_2O_5 .

Isolation of glycine and radioactivity measurements. Glycine was isolated as 2:4-dinitrophenylglycine (DNP-glycine) from TCA extracts of liver and serum and from hydrolysates of GSCu, total serum protein and fibrinogen, by Campbell & Work's (1952) method. To the isolated DNP-glycine, the amount of which was estimated with the aid of a Beckman spectrophotometer as described by Krol (1952), a measured amount of inactive DNP-glycine was added. After evaporation of the ether *in vacuo* the DNP-glycine obtained was recrystallized from ethyl acetate-light petroleum. The crystalline material, about 20 mg., was mounted on polythene disks of 1 cm.² area. All samples were counted long enough to reduce the counting error to less than 3%. A standard consisting of ¹⁴C-labelled Perspex (poly[¹⁴C]-methyl methacrylate), supplied by the Radiochemical Centre, Amersham, was used to check the counting system.

Estimation of glutathione. This was done by the manometric, methylglyoxalase method described by Woodward (1935), acetone-yeast prepared according to Albert, Buchner & Rapp (1902) being used as the source of methylglyoxalase. The concentration of liver GSH was determined by comparing the volumes of CO₂ produced by 0.125 and 0.250 ml. samples of the neutralized sulphosalicylic extract of each liver, corresponding respectively to 25 and 50 mg. of this organ, with the amount of CO₂ produced by the same volumes of the GSH standard, corresponding to 40 and 80 µg. of GSH respectively, which were run simultaneously. For the calculation of the GSH concentration, we first computed the volume *v* which should be added to, or subtracted from, the average volume of standard (0.1875 ml.)

in such a way that the average standard response (CO₂ production) would equal the average 'unknown' response, with the aid of the following equation:

$$v = \frac{I[A_{u_1} + A_{u_2} - (A_{s_1} + A_{s_2})]}{A_{s_2} + A_{u_2} - (A_{s_1} + A_{u_1})},$$

in which *I* is the interval between samples in the volume scale; *A_{u₂}* and *A_{u₁}* are the recorded values of CO₂ production for the larger and smaller samples of liver extract; *A_{s₂}* and *A_{s₁}* are the observed values of CO₂ production for the corresponding samples of standard. It can be seen that the volume *V* of standard that should give the same answer as the average unknown is obtained from

$$V = 0.1875 \pm v.$$

Since 37.5 mg. of liver is the average of the two doses of unknown, and 60 µg. the average weight of the standard, the concentration of GSH in mg./100 g. of liver can be calculated from

$$\frac{60 \times V \times 100}{0.1875 \times 37.5}.$$

The use of both 'standard' and 'unknown' at two dose levels was adopted in order to reduce the errors caused by daily changes in position and slope of the curve, which we found to occur in the dose-effect relationship between the CO₂ produced and the GSH concentration. Since it became difficult to handle all the rats in the same day, in order to control the daily variation of the rats we used one randomized representative of each group in every assay.

RESULTS

Specific radioactivity of free serum glycine

In the adrenaline- and in the saline-treated rats, the rate of fall of the specific radioactivity of the free glycine changed very much with time (Table 1). In both groups, 10 min. after the administration of [¹⁴C]glycine, the specific radioactivity of serum glycine was about twice what it was 10 min. later. Thereafter, however, this 'halving time' increased constantly in both groups, being about 60 min. between the first and the second hours, and 120 min. between the second and fourth hours after glycine administration. This shows that a single exponential curve cannot be adapted to the values for specific radioactivity of serum glycine presented in Table 1. By applying the method of Solomon (1949), we found that the following 'two compartment' equation gives a good agreement between expected and found values for the specific radioactivity of free glycine of both groups (Table 1):

$$f(t) = 100050e^{-0.099t} + 11555e^{-0.0066t}, \quad (1)$$

in which *f(t)* is the expected radioactivity of free glycine, expressed as DNP-glycine (counts/min./cm.², at infinite thickness), at time *t*.

Specific radioactivity of free liver glycine

The specific radioactivity of liver glycine of adrenaline-treated rats was substantially the same as that of the saline-treated ones at the corresponding time intervals after the administration of

[α - 14 C]glycine (Table 1). While this indicates that adrenaline did not change the permeability of liver to glycine, it is noteworthy that the rate of penetration of glycine into the liver seems to be very short, since 10 min. after glycine administration the radioactivity of liver glycine had equalled that of serum glycine. We tried to fit to our data a theoretical curve obtained by integration of a differential equation, presented previously (Henriques, Henriques & Neuberger, 1955) to describe the relation between the specific radioactivity of a product and its precursor for the case in which the former is involved in some other reaction than its reversible transformation into the precursor

$$\frac{1}{r} \frac{dx}{dt} = \frac{p_1}{r} \left[f(t) - (1+K) \frac{x}{r} \right], \quad (2)$$

in which x is the amount of radioactivity present in liver glycine; r is the size of the pool of liver glycine; p_1 is the rate of penetration of glycine into liver; $f(t)$ is the specific activity of the precursor of liver glycine (we assume plasma glycine to be the precursor of liver glycine since the radioactivity comes from the former); K is a constant. After replacing

$f(t)$ by equation (1) and integrating, we found that, with the derived theoretical equation, the best fit was obtained with a value of 1.30 for $(1+K)$ and a renewal time (r/p_1) of 5 min. for liver glycine, which is about one-third of that found previously for rabbit liver (Henriques *et al.* 1955). Accordingly, it can be seen (Table 1) that good agreement between the expected and found values for the specific radioactivity of liver glycine from adrenaline-treated and control rats was obtained with the aid of the following equation:

$$x/r = 124363 (e^{-0.099t} - e^{-0.26t}) + 9120 (e^{-0.0066t} - e^{-0.26t}), \quad (3)$$

in which x/r is the specific radioactivity of liver glycine expressed as DNP-glycine (counts/min./cm.² at infinite thickness).

Specific radioactivity of glutathione-glycine in the liver

The specific activity of GSH glycine from the adrenaline-treated as well as from the control rats rises sharply at first, reaches a maximum at the end of 60 min., falls rapidly in the next 60 min. and more slowly from then on (Table 2). However, the values for specific activity of the GSH glycine from the

Table 1. Comparison of the specific radioactivity of free glycine, as DNP-glycine, from liver and serum from rats injected with [α - 14 C]glycine (2.5 μ C/100 g. of body wt.) and adrenaline (1 μ g./g. of body wt.), with that of rats injected with [α - 14 C]glycine and saline

Each figure represents an average of 8 rats. Adrenaline was injected 2 hr. before labelled glycine. The expected values for serum in column 4 were calculated from eqn. 1, and for liver in column 7 from eqn. 2.

Time after intravenous injection of [α - 14 C]glycine (min.)	Specific radioactivity of DNP-glycine (counts/min./cm. ²) from					
	Serum			Liver		
	Adrenaline treated	Saline treated	Expected values	Adrenaline treated	Saline treated	Expected values
10	48070	49660	48070	49070	44360	44748
20	24500	23480*	24500	20940	19940	24394
60	8750	11170	7850	5800	5730	6458
120	4384	4569	5778	1805	2027	4514
180	3730	3660	3552	2280	2560	2776
240	2640	2593	2388	1214	1017	1867

* This figure represents the average value from 5 rats.

Table 2. Comparison of the specific activities of DNP-glycine samples obtained by hydrolysis of plasma fibrin, serum mixed proteins and liver glutathione (GSH) of rats injected with adrenaline (1 μ g./g. of body wt.) and [α - 14 C]glycine, with those of controls injected with saline and [α - 14 C]glycine (2.5 μ C/100 g. of body wt.)

Each figure represents an average value from 8 rats.

Time after injection of [α - 14 C]glycine (min.)	Specific radioactivity of DNP-glycine (counts/min./cm. ²) from					
	Plasma fibrinogen		Serum proteins		Liver GSH	
	Adrenaline treated	Saline treated	Adrenaline treated	Saline treated	Adrenaline treated	Saline treated
10	0	3.8	3.0	10	3860	2560
20	57	30	45	6	6220	3600
60	990	590	586	490	7440	5530
120	1539	1211	810	830	3456	3206
180	1590	1100	850	890	3760	3800
240	1572	943	870	720	1948	2072

adrenaline-treated animals were respectively 51, 73 and 34 % higher than those of the controls 10, 20 and 60 min. respectively after administration of isotopic glycine; the later values were substantially the same for both groups. Since the specific activity of free liver glycine, the presumed precursor of liver GSH glycine, was the same in both groups (Table 1), this difference in the GSH glycine curves can be explained by the reduction in the turnover time of GSH caused by adrenaline treatment. This was calculated by the graphic method of Zilversmit, Entenman & Fishler (1943), the ascending part of the curve (10–60 min.) being used, and was found to be 111 min. for the adrenaline-treated rats and 163 min. for the saline-treated ones. Very similar renewal times, when used in the equation below, were found to give a reasonable agreement between expected and found values (Fig. 1) for the specific radioactivity of GSH glycine, namely 100 min. and a K_3 value of 0.40 for the adrenaline-treated and 150 min. and a K_3 of 0.50 for the control rats:

$$\frac{u}{r_3} = \frac{p_3}{r_3} \left[\frac{124363(e^{-0.009t} - e^{-B_3t})}{B_3 - 0.009} + \frac{9120(e^{-0.0066t} - e^{-B_3t})}{B_3 - 0.0066} + \frac{124363 + 9120}{B_3 - 0.26} (e^{-B_3t} - e^{-0.26t}) \right], \quad (4)$$

in which u/r_3 is the expected specific radioactivity of GSH glycine, p_3/r_3 is the inverse of the GSH turnover time and $B_3 = p_3/r_3 (1 + K_3)$. Equation (4) was obtained by integration of equation (2) after replacing $f(t)$ by equation (3), which gives the expected activity curve for liver-free glycine, the presumed precursor of GSH glycine; other symbols of equation (2) were also changed to avoid ambiguity.

These calculations assume that the concentration of GSH does not change during the experiment, an assumption which seems to be correct (see Table 3). Indeed, although adrenaline treatment decreased slightly the concentration of liver glutathione, the lowest value found, 85 % of the control 2.5 hr. after adrenaline treatment, was not found to be significantly different from the concentration of liver GSH of the controls. It seems therefore that the adrenaline treatment decreases markedly the turnover time of liver GSH.

Incorporation of labelled glycine into the plasma fibrinogen and serum proteins

Plasma fibrinogen concentration was changed very little by adrenaline treatment during the experiment (Table 4), the maximal difference being a 20 % increase in relation to the controls at the end of 360 min. after adrenaline injection (240 min. after administration of isotopic glycine). However,

Fig. 1. Changes of the specific radioactivities of glycine obtained by hydrolysis of liver glutathione (Table 2) from adrenaline-treated (○) and control rats (●). The theoretical curves were obtained according to equation (4), a ‘renewal time’ of 100 min., $K_3=0.40$, being used for the adrenaline-treated rats (A) and 150 min., $K_3=0.50$, being used for the controls (B).

Table 3. Liver-glutathione concentration of rats after subcutaneous injection of 1 µg. of adrenaline/g. of body wt., as compared to normal controls

The time given in column 1 is the interval between the injection of adrenaline and the killing of rats. Five rats per group were used.

Time (min.)	Liver glutathione (mg./100 g. ± S.E.)	P related to controls
0	179 ± 7.9	—
60	177 ± 9.3	0.6–0.7
150	152 ± 15.6	0.1–0.2
250	156 ± 14.7	0.2–0.3

Table 4. Plasma-fibrinogen concentration of rats after subcutaneous injection of 1 µg. of adrenaline/g. of body wt., as compared to saline controls

The time given in column 1 is the interval between the injection of adrenaline or saline and bleeding of the rats. In all rats [α - 14 C]glycine was injected 2 hr. after the adrenaline. Each figure represents an average value from 8 rats.

Time (min.)	Plasma fibrinogen (mg./100 ml. ± S.E.)	
	Adrenaline-treated	Saline-treated
130	228 ± 11.0	218 ± 7.0
140	*245 ± 18.0	222 ± 8.1
180	227 ± 9.4	202 ± 5.9
240	208 ± 10.5	207 ± 5.7
300	240 ± 5.6	206 ± 17.4
360	241 ± 4.8	199 ± 11.1

* This figure represents the average value from 5 rats.

except for the 10 min. group, for which the low recorded countings were affected by large errors, the specific radioactivity of fibrinogen glycine in the adrenaline-treated rats was higher than in the control groups (Table 2) throughout the experiment (90, 68, 27, 45 and 67 % higher than the controls, 20, 60, 120, 180 and 240 min., respectively, after the administration of isotopic glycine). This could be explained by an adrenaline-induced increase in fibrinogen synthesis, which we should be able to measure by means of the turnover time of fibrinogen, since the already mentioned data for fibrinogen concentration showed very little deviation from the steady state. By the use therefore of the ascending part of the time-activity curve (20–180 min.) of fibrinogen glycine, and by assuming free glycine to be the immediate precursor of fibrinogen glycine, we calculated the turnover time of fibrinogen in both groups by the graphic method of Zilversmit *et al.* (1943), and found turnover times of 7 hr. and 9 hr. 44 min. respectively for adrenaline-treated and control rats. These figures can be accepted only for comparison, owing to the complex nature of the reactions leading to fibrinogen synthesis and release into the circulation.

It can be inferred from Table 2 that, except for the initial times, 10 and 20 min., for which determinations of specific activities are expected to be affected by large errors, the specific activity of the glycine isolated from the mixed serum proteins of the adrenaline-treated agreed within 20 % with that of the control rats.

DISCUSSION

Influence of adrenaline on the turnover of liver glutathione

The figures for the turnover of liver GSH were obtained on the assumption that liver glycine is the immediate precursor of GSH glycine. This might be true since Snoke & Bloch (1952) showed that the reaction between γ -glutamylcysteine and glycine is the last step in the synthesis of GSH, which is catalysed by extracts of acetone-dried pigeon liver. But, as Fodor, Miller, Neidle & Waelsch (1953) showed that the synthesis of GSH by extracts of sheep kidney involves the reaction between γ -glutamyl peptides and cysteinylglycine, an accurate determination of the 'renewal time' of liver GSH would require a thorough analysis of the time-activity curves of liver glycine, the glycine of cysteinylglycine and GSH glycine, although Fodor *et al.* (1953) did not show that γ -glutamyl peptides occur in the organism. It seems, however, that our data on GSH turnover can be accepted for comparison since cysteinylglycine has not so far been isolated from any biological source (Fromageot, 1953; Synge, 1953), although Tishkoff, Zaffaroni &

Tesluk (1948) presented some evidence for the existence of cysteine-containing peptide(s) in purified liver extract. It would seem therefore that cysteinylglycine occurs in the liver in small concentrations; hence, if it were an important precursor of GSH glycine, its turnover should be very quick so that its time-activity curve should closely follow that of glycine. These considerations and the already-mentioned good agreement between expected and found values show that the calculations of turnover times for liver GSH are quite close to reality and that adrenaline does indeed increase the turnover rate of GSH. Assuming a concentration of GSH of 170 mg./100 g. of liver, the rate of synthesis and breakdown (in mg./100 g. of liver/hr.) was about 60 in the controls and 90 in the adrenaline-treated rats.

Influence of adrenaline on the incorporation of labelled glycine into plasma fibrinogen

When applying the graphical method of Zilversmit *et al.* (1943) and assuming free glycine to be the immediate precursor of fibrinogen glycine, we conclude from our results that adrenaline shortens the turnover time of fibrinogen. However, since our data show that during the experimental period the fibrinogen from the adrenaline-treated rats deviated slightly from the 'steady state' the above conclusion may not be totally valid. Therefore it should be mentioned that in an experiment made with 2 groups of 10 rats each, we observed a threefold increase in fibrinogen concentration in the group treated with a high dose of adrenaline. Thus the average concentration in plasma of the rats injected subcutaneously with 400 μ g. of adrenaline (divided into 4 doses at 1.5 hr. intervals)/g. of body wt. was 620 mg./100 ml. of plasma 16 hr. after the first injection of adrenaline, whereas that of the controls was 200 mg./ml. This threefold rise in fibrinogen might be due to a complete inhibition of fibrinogen breakdown, the rate of its synthesis remaining constant, but if this were the case, the turnover time of rat fibrinogen should be about 5 hr. It is therefore suggested that, at least with high doses, the adrenaline-induced rise in fibrinogen must be partially due to an increase in fibrinogen synthesis.

SUMMARY

1. The incorporation of [α - 14 C]glycine into liver glutathione, plasma fibrinogen and mixed serum proteins was measured at times ranging from 130 to 360 min. after subcutaneous injections of adrenaline and saline into rats.

2. The 'renewal times' of glutathione were calculated to be about 100 min. in the adrenaline-treated and 150 min. in the control rats.

3. The specific radioactivity of the glycine isolated from fibrinogen of the adrenaline-treated rats was higher than in the controls throughout the experiment.

4. The specific radioactivity of the glycine isolated from mixed serum proteins of adrenaline-treated rats was very similar to that of glycine isolated from the same proteins of the saline controls at all times.

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Oxidation of *p*-Cresol and Related Compounds by a *Pseudomonas*

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Phenolic compounds are oxidized, and hence removed from industrial effluents, by micro-organisms during percolation through sewage beds. Compared with purely chemical processes of extraction this method is cheap; and the problems of effluent disposal of an expanding coal-gas industry have led to increasing interest in the mechanisms by which phenols are biologically oxidized. The present investigations on *p*-cresol afford the first detailed study of the oxidation by bacteria of methyl groups of aromatic compounds; and the extension of these studies to oxidation of xlenols has provided information concerning the specificity of such mechanisms. From observations on 'simultaneous adaptation' (sequential induction) of whole cells, and isolation of *p*-hydroxybenzoic acid from a culture of *Pseudomonas* sp. growing at the expense of *p*-cresol, Smith, Jones & Evans (1952) have suggested that *p*-cresol is oxidized by way of *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid.

EXPERIMENTAL

Organism. The organism was isolated from liquid that had circulated through an experimental plant for the treatment of gas-works effluent in the Department of Coal Gas and

Fuel Industries in this University. Dr G. M. Williamson kindly examined the organism and identified it as a species of *Pseudomonas*. It is a Gram-negative rod about $1\mu \times 2\mu$ with 2-6 polar flagella; it does not produce a pigment. From observations on its growth in various media, its biochemical activities and its behaviour towards penicillin, Terramycin and 2:4-diamino-6:7-di-isopropylpteridine it is closely related to, but not identical with, *Pseudomonas cruciviae* (Bergey, 1950). The organism was maintained by monthly serial subcultures on nutrient-agar slopes and cultures were grown with forced aeration at 30° in a medium adjusted with NaOH to pH 7, and containing, per litre: KH_2PO_4 , 2 g.; $(\text{NH}_4)_2\text{SO}_4$, 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; *p*-cresol, 0.3 g. The relationship between bacterial crop and *p*-cresol concentration was approximately linear up to 0.3 g. of *p*-cresol/l., but at concentrations greater than 0.4 g./l. the compound was increasingly inhibitory to growth. When 25 ml. of liquid medium was inoculated from the slope, full growth was reached in 2 days. The whole of this culture was used as inoculum for 1 l. of fresh medium and after overnight growth the new culture was, in turn, used to inoculate 10 l. of medium; the cells were harvested after further growth for 18 hr. All buffer solutions contained KH_2PO_4 of the strength stated in the text, adjusted to pH 7 by addition of NaOH. For measurements of O_2 uptake, cells were washed twice with 0.1 M-phosphate buffer and suspended in this buffer to give a cell density of 10 mg. dry wt./ml. Cell-free extracts were prepared with the Hughes bacterial press (Hughes, 1951), without abrasive, at -14°. Each 1 g. of