oxidation of glutamine results in the formation of aspartic acid.

2. In the presence of substances giving rise to acetyl-coenzyme A, the rate of oxidation of glutamine is decreased, but the extent of oxidation is increased.

3. The rate of glutamine oxidation is increased by sodium hydrogen carbonate and α -oxo acids.

4. The incorporation of amino acids into proteins is inhibited under anaerobic conditions by pyruvate, oxaloacetate and phenylpyruvate. This inhibition is reversed specifically by glutamine.

5. The incorporation of amino acids into proteins is inhibited under aerobic conditions by phenylpyruvate, and this inhibition is reversed by glutamine, pyruvate, oxaloacetate and glucose.

6. Glutamine may serve as a source of energy under certain circumstances for the incorporation of amino acids into proteins by these cells.

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Biochemical Changes in the Tissues of Animals Injected with Iron

3. LIPID PEROXIDATION*

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The presence in tissues of acid-fast polymers, composed of condensation products of peroxidized lipid with protein, is characteristic of a number of experimental and clinical conditions, of which old age is by far the most common. Lipofuscin polymers of apparently similar type are readily produced in animals injected with massive doses of

iron-dextran (Golberg, Smith & Martin, 1957), and constitute part of the evidence for a relative lack of antioxidant capacity accompanying tissue siderosis. In the rat this effect is manifested as vitamin E deficiency, accentuated by an inadequate supply of dietary tocopherol or by an excessive proportion of unsaturated fat, but not by lack of vitamin A (Golberg & Smith, 1958, 1960).

The biosynthesis of acid-fast polymers depends on the initial formation of auto-oxidized unsaturated lipids (Casselman, 1951). We have pre-

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viously put forward histochemical and biochemical evidence of peroxidized lipids in the adipose tissue of rats in which massive siderosis had been induced by means of iron-dextran (Golberg & Smith, 1958). This and related aspects of iron-overloading have been investigated further, the thiobarbituric acid reaction being used as a measure of lipid 'peroxides' (Wolfson, Wilbur & Bernheim, 1956). In fact the method estimates tissue-breakdown products of lipid peroxides, notably malonaldehyde (Kitabchi, McCay, Carpenter, Trucco & Caputto, 1960), and has been strongly criticized for its lack of specificity by Green, Diplock, Bunyan, Edwin & McHale (1961). Nevertheless, it provides a quantitative approach which has yielded useful information.

METHODS

Dosage with iron-dextran and other macromolecular preparations. An iron-dextran complex (Imferon) was used as described by Golberg, Martin & Batchelor (1960a). The injection routes and doses are stated in Tables 1-4. Low-molecular-weight dextran has been described by Golberg *et al.* (1960a). Aluminium-dextran complex contained 18.8 mg. of aluminium/ml. This preparation was deposited at the site of injection and, apart from a small amount taken up by macrophages on the periphery of the injection mass, no aluminium could be detected histochemically in liver or other organs. Antimony-dextran complex containing 14.5 mg. of quinquevalent antimony/ml. was widely distributed systemically.

Reagents. α -Tocopheryl acetate was obtained from Roche Products Ltd., ATP and NAD from C. F. Boehringer und Soehne, and D-glucuronolactone from L. Light and Co. Ltd.

Preparation of homogenates. The method of killing the animals and removing the organs has been described (Golberg *et al.* 1960a). Tissues for 'peroxide' determinations were homogenized in cold 0.07M-phosphate (Na_2HPO_4 - KH_2PO_4) buffer, pH 7.4, in an all-glass homogenizer. Homogenate concentrations were 1 g. wet wt./15 ml. of phosphate buffer. Liver for fractionation (1 g.) was homogenized in 5 ml. of 0.15M-potassium phosphate buffer, pH 7.5, in a glass homogenizer with a Teflon pestle.

Preparation of cytoplasmic fraction. The small particle and supernatant fractions were obtained by centrifuging liver homogenates at 5200g (4°) for 10 min. as described by Caputto, McCay & Carpenter (1958). A sample of each cytoplasmic fraction was homogenized in a small glass homogenizer with sufficient α -tocopheryl acetate to give a concentration of 5 mg./ml. All assays were carried out on 0.25 ml. of cytoplasmic fraction.

Estimation of thiobarbituric acid reactants. The method of Wolfson *et al.* (1956) was used. Tissue homogenate (1 or 2 ml.) in stoppered tubes was incubated at 37° with shaking for 2 hr. Ascorbic acid in solution (0.05 ml.) was added when required. After incubation, 1 vol. of 20% trichloroacetic acid and 2 vol. of 0.67% 2-thiobarbituric acid were added. The tubes were heated for 15 min. at 100° , cooled and centrifuged. The supernatant fluids were diluted with 3 vol. of water and the extinction was measured at 530 m μ in 1 cm. cells. When cytoplasmic fraction was used, 0.25 ml. was diluted with 0.75 ml. of 0.15M-phosphate

buffer, pH 7.5, and treated in the same way as 1 ml. of homogenate. Extinction of the supernatant was read without dilution.

Ascorbic acid synthesis. The method of Carpenter, Kitabchi, McCay & Caputto (1959) was used. The substrate, sodium glucuronate, was prepared from glucuronolactone by addition of 1 equiv. of NaOH. The final volume of the assay mixture was 1 ml. The reaction was stopped by the addition of 1 ml. of 20% (w/v) trichloroacetic acid and ascorbic acid was determined by the method of Roe & Kuether (1943).

RESULTS

Effect of iron-dextran on lipid 'peroxides' in tissues. Material reacting with thiobarbituric acid was estimated in tissue homogenates from rats and mice given iron-dextran (Table 1). There was a striking increase at the injection site in the thigh muscles of iron-treated mice, with a parallel increase in the overlying skin and subcutaneous tissues, as well as in the liver. Even the uninjected hind-limb muscle and skin showed a significant increase, indicating the widespread nature of the changes induced by the generalized siderosis. The prolonged duration of these changes was established by similar measurements carried out in rats that had been given iron-dextran almost a year earlier. Muscle, skin, liver and kidney had amounts of thiobarbituric acid reactants that were greater ($P < 0.001$) than those of untreated control rats; amounts in heart and brain were apparently unaffected by treatment with iron. Incubation for 2 hr. increased the 'peroxide' values, the differences between them being maintained.

The importance of the dose of iron used was demonstrated in experiments involving the administration to mice of the human therapeutic dose of iron-dextran, calculated on a basis of body weight (Table 2). Under these circumstances there was no significant increase in lipid 'peroxide' of mouse muscle or skin, but in the rat the values were increased. No such effect was seen in liver and kidney of either species.

Relation of ascorbic acid to lipid 'peroxides' in tissues. Aged untreated rats had initial amounts of 'peroxide' in the skin, muscle, liver and kidney that were similar to those found in the corresponding tissues of younger rats. The amounts increased on incubation, substantially so in liver (Table 3). Incubation in the presence of ascorbic acid (0.2 mg./ml.) greatly enhanced the formation of 'peroxide'. Iron-treated rats of the same age showed high initial amounts of 'peroxide', which increased somewhat on incubation to attain values considerably above those reached by the incubated tissue homogenates from untreated rats. Ascorbic acid at a concentration of 0.2 mg./ml. greatly increased 'peroxide' formation in liver and kidney; at 1.0 mg./ml. it had no stimulating effect.

It was of interest also to gauge the influence of endogenous ascorbic acid by following its synthesis in liver. There was no significant difference between the initial ascorbic acid contents of liver homogenates from the untreated and iron-treated groups of mice or rats. In the mice, which had received iron-dextran as recently as 1 week before, there was no effect on total ascorbic acid synthesis by the whole liver. In rats last treated with iron 29 weeks earlier, synthesis was doubled (Table 4). Addition

of α -tocopheryl acetate to the incubation mixture only affected siderotic rat liver, where synthesis of ascorbic acid was more than doubled. The antioxidant function of the added tocopherol was reflected in the 'peroxide' values after incubation, which incidentally afforded evidence that lipid 'peroxidation' is responsible for the increase in material reacting with thiobarbituric acid.

Specificity of the iron effect. Low-molecular-weight dextran, antimony-dextran and aluminium-

Table 1. *Thiobarbituric acid reactants present in tissues from untreated and iron-treated mice and rats*

Repeated intramuscular injections of iron-dextran were given into one hind limb of male mice (eight daily doses, each of 5 mg. of iron) and male rats (eleven doses, given three times weekly, each of 75 mg. of iron/kg.). The mice were killed 2 weeks, and the rats 24 weeks, from the start. In the iron-treated mice, 'uninjected' skin and muscle were taken from the opposite hind limb. 'Skin' includes subcutaneous tissues. Average wet weight of tissue used was 67 mg./ml. 'Reactants' refers to thiobarbituric acid reactants, expressed as E_{530} /g. of tissue, measured before and after incubation at 37° for 2 hr. The values are means $\pm$ S.D.

Tissue	Group	Mice			Rats		
		No.	Reactants		No.	Reactants	
			Before	After		Before	After
'Skin'	Untreated	6	3.6 $\pm$ 0.7	7.2 $\pm$ 1.1	9	1.8 $\pm$ 0.1	4.2 $\pm$ 1.0
	Iron-treated	6	17.4 $\pm$ 1.9	29.4 $\pm$ 5.5	9	12.0 $\pm$ 2.3	14.4 $\pm$ 2.0
	Uninjected	6	7.8 $\pm$ 0.8	12.0 $\pm$ 1.4	—	—	—
Muscle	Untreated	6	4.8 $\pm$ 0.7	10.2 $\pm$ 0.8	9	1.8 $\pm$ 0.4	5.4 $\pm$ 0.8
	Iron-treated	6	17.4 $\pm$ 2.8	28.8 $\pm$ 6.1	9	15.6 $\pm$ 3.2	20.4 $\pm$ 5.2
	Uninjected	6	8.4 $\pm$ 0.9	16.2 $\pm$ 1.5	—	—	—
Liver	Untreated	6	3.5 $\pm$ 0.5	25.8 $\pm$ 6.7	5	3.6 $\pm$ 0.3	12.0 $\pm$ 2.2
	Iron-treated	6	7.8 $\pm$ 0.7	48.6 $\pm$ 4.2	6	10.8 $\pm$ 1.0	16.8 $\pm$ 2.4
Kidney	Untreated	—	—	—	5	3.6 $\pm$ 0.6	6.6 $\pm$ 1.3
	Iron-treated	—	—	—	6	9.6 $\pm$ 1.6	14.4 $\pm$ 0.8
Heart	Untreated	—	—	—	5	2.8 $\pm$ 1.2	8.4 $\pm$ 1.8
	Iron-treated	—	—	—	6	4.4 $\pm$ 1.8	8.0 $\pm$ 1.2
Brain	Untreated	—	—	—	5	5.3 $\pm$ 1.2	26.4 $\pm$ 1.2
	Iron-treated	—	—	—	6	7.2 $\pm$ 3.0	31.8 $\pm$ 4.2
Testis	Untreated	—	—	—	3	4.4 $\pm$ 0.9	13.2 $\pm$ 0.7
	Iron-treated	—	—	—	3	8.4 $\pm$ 0.7	13.8 $\pm$ 0.6

Table 2. *Effect of treatment with moderate doses of iron-dextran*

Repeated intramuscular injections of iron-dextran (six daily doses of 5 mg. of iron/kg.) were given into one hind limb of each of six mice or rats; they were killed 3-9 days after the last dose. 'Uninjected' skin and muscle were taken from the other hind limb of each animal. Skin includes subcutaneous tissues. Values are means $\pm$ S.D.

Tissue	Group	Thiobarbituric acid reactants (E_{530} /g. of tissue)	
		Mice	Rats
'Skin'	Untreated	3.6 $\pm$ 1.3	2.4 $\pm$ 0.4
	Iron-treated	3.6 $\pm$ 0.5	10.2 $\pm$ 1.3
	Uninjected	3.6 $\pm$ 0.9	8.4 $\pm$ 1.3
Muscle	Untreated	3.6 $\pm$ 1.1	1.8 $\pm$ 0.4
	Iron-treated	3.6 $\pm$ 1.0	21.0 $\pm$ 2.8
	Uninjected	2.4 $\pm$ 0.5	8.4 $\pm$ 3.4
Liver	Untreated	2.8 $\pm$ 0.4	2.4 $\pm$ 0.6
	Iron-treated	1.8 $\pm$ 0.4	1.8 $\pm$ 0.3
Kidney	Untreated	3.0 $\pm$ 0.5	3.6 $\pm$ 0.5
	Iron-treated	2.4 $\pm$ 0.2	2.4 $\pm$ 0.4

dextran complexes were given to mice under conditions comparable with those in which iron-dextran has a striking effect on tissue 'peroxides'. With dextran itself there was no difference from untreated control mice (Table 5). Lipid 'peroxide' was slightly but significantly increased by antimony-dextran in muscle from the injection site and in liver after incubation ($P < 0.01$ and < 0.02 respectively). The only distinct result of aluminium-dextran was to decrease lipid 'peroxide' in injected muscle; more striking was the inhibition of the formation of the usual large increment on incubation. The same effect was apparent when mice were given only three or six injections of aluminium-dextran. In these groups there was also inhibition of 'peroxide' formation on incubation of uninjected skin and muscle, and of liver (after three doses). The inhibition was demonstrable on adding aluminium-dextran to liver and muscle homogenates from

untreated mice. It was not due to interference by aluminium-dextran in the thiobarbituric acid reaction.

These studies were supplemented by experiments carried out *in vitro* with liver microsomes from untreated rats. The results (Fig. 1) with ferrous sulphate, ferric chloride and horse-spleen ferritin showed a distinct rise in thiobarbituric acid reactants when higher concentrations of ionized iron or ferritin iron were present. In addition there was a clear stimulatory effect of ascorbic acid on 'peroxide' formation, which was somewhat enhanced in the presence of higher concentrations of ionized iron, but not of ferritin. With Al^{3+} ions, even at low concentrations, agglutination of the suspended microsomes took place. Hence the small decreases in 'peroxide' must be disregarded, and the observations made above with aluminium-dextran may be due to a similar phenomenon.

Table 3. *Effect of added ascorbic acid*

Repeated intramuscular injections of iron-dextran were given into alternate hind limbs of female rats weighing 140–180 g. (22 doses, given three times weekly, each of 75 mg. of iron/kg.). The rats were killed 54 weeks from the start. Values in the first three columns are means $\pm$ S.D. for groups of six untreated with nine, nine and six iron-treated rats; values in the last column are the mean results for three rats. Incubation was at 37° for 2 hr.; average wet weight of tissue used was 67 mg./ml. 'Skin' includes subcutaneous tissues.

Concn. of ascorbic acid (mg./ml.) Group Tissue		Thiobarbituric acid reactants (E_{530} /g. of tissue)			
		Before incubation	After incubation with ascorbic acid		
			0	0.2	1.0
Untreated	'Skin'	1.9 $\pm$ 0.1	4.0 $\pm$ 1.0	15.6 $\pm$ 2.8	—
	Muscle	2.0 $\pm$ 0.4	5.3 $\pm$ 0.8	15.0 $\pm$ 3.9	—
	Liver	3.5 $\pm$ 0.3	12.0 $\pm$ 2.2	24.6 $\pm$ 2.4	—
	Kidney	3.7 $\pm$ 0.6	6.6 $\pm$ 1.3	12.6 $\pm$ 1.9	—
Iron-treated	'Skin'	12.0 $\pm$ 2.3	14.4 $\pm$ 2.0	15.6 $\pm$ 5.4	16.8
	Muscle	15.6 $\pm$ 3.2	20.4 $\pm$ 5.2	25.8 $\pm$ 10.8	22.2
	Liver	10.8 $\pm$ 1.0	16.8 $\pm$ 2.4	46.2 $\pm$ 10.2	10.8
	Kidney	9.6 $\pm$ 1.6	14.4 $\pm$ 0.8	30.0 $\pm$ 3.6	12.0

Table 4. *Ascorbic acid synthesis by liver homogenates*

Iron-dextran was given intramuscularly to male mice (eight daily doses over 2 weeks, each of 5 mg. of iron) and intravenously to female rats (10 weekly doses, each of 10 mg. of iron). The mice were killed 3 weeks, and the rats 38 weeks, from the start. Incubation was at 37° for 2 hr. Values are means $\pm$ S.D. for groups of five rats and six mice.

Species Group		α -Tocopheryl acetate (mg./ml.)	Ascorbic acid synthesis (μ moles/2 hr.)		Thiobarbituric acid reactants (E_{530} /g. of liver)	
			Per g. of liver	Whole liver	Before incubation	After incubation
Mouse	Untreated	0	1.09 $\pm$ 0.31	1.62 $\pm$ 0.57	0.9 $\pm$ 0.1	4.0 $\pm$ 0.4
	Untreated	1.25	1.15 $\pm$ 0.20	1.63 $\pm$ 0.27	—	2.7 $\pm$ 0.2
	Iron-treated	0	0.72 $\pm$ 0.30	1.71 $\pm$ 0.75	3.2 $\pm$ 0.4	13.6 $\pm$ 1.5
	Iron-treated	1.25	0.79 $\pm$ 0.35	1.85 $\pm$ 0.60	—	3.4 $\pm$ 0.5
Rat	Untreated	0	0.68 $\pm$ 0.17	3.84 $\pm$ 1.42	1.8 $\pm$ 0.2	7.6 $\pm$ 0.7
	Untreated	1.25	0.73 $\pm$ 0.29	3.83 $\pm$ 1.64	—	1.6 $\pm$ 0.2
	Iron-treated	0	0.99 $\pm$ 0.28	7.34 $\pm$ 1.94	4.0 $\pm$ 0.4	10.6 $\pm$ 0.6
	Iron-treated	1.25	1.91 $\pm$ 0.45	16.4 $\pm$ 3.70	—	4.2 $\pm$ 0.4

Table 5. *Effects of other macromolecular preparations*

Daily intramuscular injections were given into one hind limb of male mice as follows (preparation used, no. of doses and vol. of dose, days from start when each group of six mice was killed): 20% (w/v) low-molecular-weight dextran, eight 0.1 ml., 13-14 days; antimony-dextran complex (1.45% w/v, of Sb), eight 0.1 ml., 15-16 days; aluminium-dextran complex (1.88% w/v, of Al), eight 0.05 ml., 12-13 days. 'Uninjected' skin and muscle were taken from the non-injected hind limb. 'Reactants' refers to the thiobarbituric acid-reaction product, expressed as E_{550} /g. of tissue, measured before and after incubation at 37° for 2 hr. Values are means $\pm$ s.d.

Tissue	Group	Untreated		Low-molecular-weight dextran		Antimony-dextran		Aluminium-dextran	
		Reactants before	Reactants after	Reactants before	Reactants after	Reactants before	Reactants after	Reactants before	Reactants after
Skin	Untreated	1.8 $\pm$ 0.4	7.7 $\pm$ 0.9	—	—	—	—	—	—
	Injected	—	—	2.4 $\pm$ 0.4	8.1 $\pm$ 1.3	1.9 $\pm$ 0.4	9.1 $\pm$ 1.2	2.6 $\pm$ 0.2	8.7 $\pm$ 1.7
	Uninjected	—	—	2.5 $\pm$ 0.4	7.5 $\pm$ 0.8	1.8 $\pm$ 0.6	7.5 $\pm$ 1.1	2.4 $\pm$ 0.3	9.0 $\pm$ 1.2
Muscle	Untreated	2.9 $\pm$ 0.4	10.7 $\pm$ 1.9	—	—	—	—	—	—
	Injected	—	—	2.7 $\pm$ 0.3	10.1 $\pm$ 0.9	1.8 $\pm$ 0.3	14.9 $\pm$ 2.0	1.1 $\pm$ 0.3	1.6 $\pm$ 0.4
	Uninjected	—	—	2.5 $\pm$ 0.4	9.1 $\pm$ 1.0	2.0 $\pm$ 0.3	10.6 $\pm$ 0.5	2.4 $\pm$ 0.3	9.0 $\pm$ 1.2
Liver	—	3.0 $\pm$ 1.1	26.7 $\pm$ 2.6	3.9 $\pm$ 0.4	27.2 $\pm$ 2.2	2.8 $\pm$ 0.2	32.0 $\pm$ 2.6	2.4 $\pm$ 0.2	25.1 $\pm$ 3.0

Fig. 1. Effect of addition of iron with and without ascorbic acid on the contents of thiobarbituric acid reactants produced on incubation of rat-liver microsomes: ●, Fe²⁺ ions; ■, Fe³⁺ ions; ▲, ferritin (control values obtained without iron or ascorbic acid); ○, □, △, as before but with ascorbic acid added (0.2 mg./ml.). For convenience all results are plotted as percentages of the control values obtained with ascorbic acid but without added iron.

DISCUSSION

Lipid 'peroxide' in tissues

Bieri & Anderson (1960) have shown that a close relationship exists between the extent of lipid 'peroxidation' in incubated tissue homogenates and the vitamin E content of the animal's diet, so that a diet adequate for the living animal may not contain sufficient vitamin E to prevent 'peroxidation' *in vitro* of isolated tissue components. Our stock diet (Oxo 41) contains 5.2 μ g. of α -tocopherol/g., together with lesser quantities of other tocopherols (Golberg & Smith, 1958). This may be compared with the α -tocopherol contents of two diets used by Edwin, Diplock, Bunyan & Green (1961), 2.4 μ g. of α -tocopherol/g. in a vitamin-E-deficient basal diet and 9.5 μ g./g. for a nutritionally adequate stock diet. On these two diets, Edwin *et al.* (1961) found that 13-month-old female rats showed striking differences in the α -tocopherol contents (in μ g./g.) of tissues: liver (7.5, 29.9), muscle (2.1, 21.4), kidney (1.2, 25.9) and brain (1.0, 11.1). In all rats, the tocopherol content of heart was greatly reduced when the animal was on the vitamin-E-deficient diet. The same authors also noted the presence of high amounts of lipid 'peroxides' in the brains of adequately fed rats. It follows from these observations that the relatively high initial and final amounts of 'peroxide' in tissue homogenates from our untreated animals are to be attributed in part to a somewhat low tocopherol content of their diet.

Siderosis induced by iron-dextran in doses of 800–1600 mg. of iron/kg. had a striking effect on lipid ‘peroxidation’ in liver, kidney, leg muscle and skin, but not in heart or brain, in which little iron is deposited (Golberg & Smith, 1958). When a dose approaching the human clinical dose of 30 mg. of iron/kg. was given, iron-treated animals no longer had a higher amount of tissue ‘peroxide’, except in rat skin and muscle. The rat retains an exceptionally high proportion of iron at the injection site (Golberg, Martin & Smith, 1960*b*; Baker, Golberg, Martin & Smith, 1961); but the high amounts of ‘peroxide’ in ‘uninjected’ skin and muscle perhaps denote a special susceptibility to lipid ‘peroxidation’ in the rat, to which Bieri & Anderson (1960) have drawn attention.

Zalkin & Tappel (1960) have reviewed the thiobarbituric acid reactants in animal tissues and emphasized the sensitivity and lack of specificity of the reaction. The increased values found in siderotic tissues might thus be regarded as not necessarily connected with lipid ‘peroxidation’, were it not for the direct effect of added α -tocopherol in preventing an increase in thiobarbituric acid reactants on incubation and the relationship with ascorbic acid discussed below.

Formation of thiobarbituric acid reactants

In tissue homogenates, or fractions derived from them, interactions between unsaturated fatty acids, iron, vitamin E and ascorbic acid may be involved in the formation of thiobarbituric acid reactants. Non-enzymic auto-oxidation of unsaturated lipid is, in its initial stages, a reaction catalysed by trace metals and their complexes, such as iron-porphyrins (Uri, 1957). Inhibition of Fe^{2+} ion catalysis has been put forward as the mechanism of the effects on lipid ‘peroxidation’ of chelating agents (Barber & Wilbur, 1959), siderophilin and other specific vertebrate iron-binding serum proteins (Barber, 1961), and fatty acids set free by phospholipase action in the intestinal mucosa (Ottolenghi & Bernheim, 1960). In normal liver mitochondria (Thiele & Huff, 1960), and in liver microsomes from vitamin-E-deficient rats, the non-enzymic formation of thiobarbituric acid reactants is promoted by ferrous or ferric salts (Kitabchi *et al.* 1960). The antioxidant activity of vitamin E involves the inhibition *in vivo* of haematin-catalysed oxidation of unsaturated lipids (Tappel & Zalkin, 1960). Ferritin brings about the oxidation of unsaturated fatty acids *in vitro* (unpublished observations). In tissues it is probable that reducing agents mobilize Fe^{2+} ions, which are then transported by endogenous small chelator molecules (Charley, Rosenstein, Shore & Saltman, 1960). Ascorbic acid functions in both capacities (Takeda & Hara, 1955) and in both directions (Mazur, Green & Carleton, 1960).

Moreover, lipid ‘peroxide’ formation takes place by co-oxidation of ascorbic acid, both processes being mediated by a metal catalyst such as Fe^{2+} ions at low concentration (Ottolenghi, 1959).

Enzymic formation of thiobarbituric acid reactants may occur when ascorbic acid synthesis is blocked, probably through inhibition of L-gulonolactone oxidase. Such inhibition occurs in vitamin E deficiency, or, *in vitro*, on addition of ascorbic acid (Kitabchi *et al.* 1960). The inhibition is overcome by α -tocopherol acting *in vivo* or *in vitro*, by ethylenediaminetetra-acetic acid, or by manganese or cobalt salts. Vitamin E deprivation is soon reflected in a powerful effect on ascorbic acid synthesis and ‘peroxide’ formation *in vitro* (Kitabchi *et al.* 1959). Peroxide-decomposing systems of animal cells also involve ascorbic acid (Updegraff & Bovey, 1958). It follows that the maintenance of a low amount of lipid peroxide in tissues is dependent on vitamin E, both directly as an antioxidant and indirectly through its effect on ascorbic acid synthesis and destruction. Through their action on vitamin E (Golberg & Smith, 1958), massive tissue stores of iron can thus influence the state of lipid peroxidation.

The present results (Table 3) must be examined in the light of these considerations. The addition of 0.2 mg. of ascorbic acid/ml. to homogenates of untreated rat tissues brought about a striking increase in the thiobarbituric acid reactants formed on incubation. With a higher concentration of ascorbic acid (1.0 mg./ml.) the ‘peroxide’ amount on incubation was very much decreased. This effect has been observed with normal mouse-liver homogenate and occasionally also with brain (Baker, Bloom & Bland, 1959), and has been attributed to inhibition of ‘peroxide’ formation, though it might equally reflect ‘peroxide’ destruction by ascorbic acid.

A further aspect of the relationship between iron, ascorbic acid and tocopherol is seen in the figures for ascorbic acid synthesis (Table 4). Mice that had received massive doses of iron shortly before showed the expected effect on liver ‘peroxide’ but ascorbic acid synthesis remained normal when calculated for the whole hyperplastic liver (Golberg *et al.* 1960*a*). In rats that had been given iron intravenously (so that it accumulated principally in the liver), and had survived a further 7 months, there was no decrease in ascorbic acid synthesis per gram of liver; yet the addition of tocopherol approximately doubled it.

It is clear from Table 5 that the iron moiety and not the dextran in iron-dextran is responsible for the enhanced formation of thiobarbituric acid reactants. Antimony-dextran had little effect; aluminium-dextran on the other hand had a striking antioxidant action on the muscle in which

it was deposited on injection, but not in skin or liver. On the periphery of the injected mass of aluminium-dextran, small amounts are taken up by macrophages (unpublished observations), which presumably liberate some ionized aluminium. The inhibition of 'peroxide' formation is reminiscent of the action of manganese or cobalt salts in preventing the formation of malonaldehyde on incubation (Kitabchi *et al.* 1960).

Biochemical lesion in siderosis

Zalkin & Tappel (1960), among many other authors, have stressed the toxicity of lipid peroxides, attributing to them the damage caused by ionizing radiation, the degeneration of microsomes in vitamin-E-deficient animals and various metabolic derangements (Tappel & Zalkin, 1960). We have been struck by the presence of a permanently high content of thiobarbituric acid reactants, apparently lipid peroxides, at the injection site in our rats and mice, over the greater part of their life span. The development of extensive fibrosis, large amounts of lipofuscin polymers (Baker *et al.* 1961) and, in some cases, of sarcomata (Richmond, 1959; Haddow & Horning, 1960; Golberg *et al.* 1960*b*) after a series of superimposed massive injections of iron-dextran has been ascribed by Haddow (1959) to 'metal carcinogenesis'. These pathological consequences may represent a direct action of iron, but it is more likely that they are an indirect outcome of the secondary effects, including lipid peroxidation, induced by massive deposits of stored iron.

Does lipid peroxidation help to bring about pathological changes in haemochromatosis and other human siderotic states? Although we have demonstrated lipid peroxidation in siderotic animals by three different techniques, biopsies of liver and perirenal fat from a patient with haemochromatosis failed to show the presence of lipid peroxides by the histochemical method of Hartmann & Glavind (1949). The maintenance of what Bieri & Anderson (1960) have termed an 'antioxygenic potential' is of paramount importance in preventing the peroxidation of tissue lipids *in vivo*. The close association of stainable granular iron and of lipofuscin, widely distributed in the tissues of healthy old mice (Spicer, 1960) and men (Hueck, 1912; Lillie, 1954), suggests the cause and effect of failure to maintain an adequate local 'antioxygenic potential'.

SUMMARY

1. Thiobarbituric acid has been used to measure lipid 'peroxides' in tissues of mice and rats injected with iron-dextran. With high doses, an increased content of thiobarbituric acid reactants was found at the injection site in muscle and skin, and in liver and kidney. In mice, a similar change took place in

skin and muscle of the opposite, uninjected limb. The change was still present a year after injection.

2. Moderate doses of iron-dextran did not induce an increase in thiobarbituric acid reactants in the mouse but did so at the injection site of the rat, a difference attributable to high local retention of iron in the rat.

3. The addition of ascorbic acid to tissue homogenates from untreated rats increased the concentration of lipid 'peroxides' after incubation; in iron-treated rats, only liver and kidney contents were elevated. Ascorbic acid synthesis by liver homogenates was not significantly changed in iron-treated mice and rats. The addition of α -tocopherol to the test system doubled ascorbic acid synthesis in siderotic rat liver but not in mouse liver. Vitamin E also decreased thiobarbituric acid reactants, in most cases to the amount found before incubation.

4. No effect on tissue 'peroxides' comparable with that of iron-dextran was brought about by low-molecular-weight dextran, antimony-dextran or aluminium-dextran. It is suggested that the accumulation of large amounts of iron results in a failure to maintain an adequate local 'antioxygenic potential' and thus induces the formation of high contents of tissue peroxides, to which subsequent pathological changes may be attributed.

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The Synthesis of Cell Constituents from Butane-2,3-diol by *Pseudomonas* sp.

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Juni & Heym (1956) showed that the dissimilation of butane-2,3-diol by micro-organisms proceeds via a cyclic mechanism to acetate. Butane-2,3-diol is oxidized to 2-hydroxybutan-3-one and thence to diacetyl, which, in the presence of thiamine pyrophosphate, is hydrolysed to acetate and an 'active acetaldehyde' complex. The complex undergoes an instantaneous condensation with a further molecule of diacetyl to form 3-hydroxy-3-methylpentane-2,4-dione, which is then subsequently reduced to 2,3-dihydroxy-3-methylpentan-4-one; this is hydrolysed to give a second molecule of acetate and butane-2,3-diol. The net effect of one turn of this cycle is to oxidize butane-2,3-diol to acetate according to the following reaction:

Dagley (1958) suggested that the required synthesis of intermediates of the tricarboxylic acid cycle might occur via the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957).

The main purpose of this paper is to show that *Pseudomonas* sp. (tentatively identified as *P. fluorescens*), utilizing butane-2,3-diol as sole source of carbon, incorporates [$1\text{-}^{14}\text{C}$]acetate into cell constituents consistent with the operation of the tricarboxylic acid and glyoxylate cycles. No evidence

has been obtained in support of the direct formation of succinate from acetate (Thunberg, 1920; Glasky, Eicholz & Rafelson, 1958; Glasky & Rafelson, 1957, 1959).

This work has been presented in part to the Biochemical Society (Hullin & Hassall, 1960).

MATERIALS AND METHODS

Maintenance and growth of the organism. The organism was originally isolated from soil, by S. Dagley, on account of its ability to utilize butane-2,3-diol as sole source of carbon for growth. Stock cultures were kept on agar slopes consisting of (w/v): KH_2PO_4 , 0.2%; $(\text{NH}_4)_2\text{SO}_4$, 0.2%; thiamine hydrochloride, 0.001%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04%; butane-2,3-diol, 0.1%; agar, 2.0%; the pH was adjusted to 7.0–7.2 with NaOH. The organism was subcultured every 10–12 weeks, grown at 30°, and stored at 2°; desiccation of the slopes was prevented by sealing the tubes with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2).

Cells were grown for 18 hr. with forced aeration at 30° in 10 l. flasks, containing 9 l. of medium, after inoculation with 800 ml. of a culture grown overnight in the same medium. This medium contained (g./l.): KH_2PO_4 , 5.0; $(\text{NH}_4)_2\text{SO}_4$, 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; butane-2,3-diol, 1.8; the pH was adjusted to 7.0–7.2 with NaOH. Cell concentrations were determined turbidimetrically in a Hilger Spekker photoelectric absorptiometer fitted with Ilford filters neutral H. 508 and blue O.B.₂. The cells were harvested while still in the logarithmic phase of growth (0.25–0.35 mg. dry wt./ml.) using a Sharples continuous-flow centrifuge.

Manometric studies of oxygen uptake. The conventional Warburg respirometer was used, as described by Umbreit,

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