Nitrate Reductase and Related Enzymes in *Escherichia coli*

By C. F. HEREDIA AND A. MEDINA*

Sección de Bioquímica Vegetal del Instituto de Edafología y Fisiología Vegetal, Departamento de Enzimología, Centro de Investigaciones Biológicas, C.S.I.C., Madrid, Spain

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Several reports about nitrate reduction in *Escherichia coli* have appeared during the last few years. Taniguchi, Sato & Egami (1956) have found in cell-free extracts of *E. coli*, grown in a peptone broth-agar medium containing nitrate, a particulate system which reduces nitrate to nitrite, only under anaerobic conditions and with reduced diphosphopyridine nucleotide, formate or reduced methylene blue as electron donors and flavin nucleotides as electron carriers. Wainwright (1955) found that nitrate-reductase activity in *E. coli*, strain 1431, was enhanced in anaerobiosis by vitamin K₃ and that the maximal activity was obtained when both vitamin K₃ and flavin nucleotides were added to the incubation mixtures. Cheniae & Evans (1958, 1959) have also observed an activation by vitamin K₃ of nitrate reductase by preparations of *Rhizobium japonicum* when reduced diphosphopyridine nucleotide was the donor. Nicholas & Nason (1955) found in cell-free extracts of *E. coli* grown in a synthetic medium a soluble, flavin-dependent diphosphopyridine nucleotide-nitrate reductase which reduces nitrate to nitrite aerobically. Medina & Heredia (1958) found that cell-free extracts of *E. coli*, grown either in a synthetic medium or in a complex broth medium, contain a reduced diphosphopyridine nucleotide-dependent system which reduces nitrate to nitrite aerobically with vitamin K₃ as electron carrier without any apparent participation of flavin in the process.

The work presented in this paper shows that nitrate reduction takes place under aerobic as well as under anaerobic conditions and in both cases is catalysed by a menadione-reductase-nitrate-reductase system which uses vitamin K₃ as electron carrier. In addition to this pathway, nitrate can also be reduced to nitrite, under completely anaerobic conditions, through an apparently flavin-dependent system, presumably related to the reduced diphosphopyridine nucleotide-oxidase system.

MATERIALS AND METHODS

Preparation of cell-free extracts. Homogenates were prepared from cells of *E. coli*, strain 86 N.C.T.C. (Oxford),

grown aerobically in a synthetic medium (Nicholas & Nason, 1955) or in a peptone-beef-extract broth medium containing 0.5% of KNO₃. Round flasks of 3 l. capacity, containing 1.5 l. of medium, were inoculated and allowed to stand at 37° for about 12 hr. Cells were harvested by filtering through Chamberland filters, Pasteur-system type 1F-NP and centrifuging in the cold at 8000 g for 5 min. The cells were washed twice on the centrifuge with 0.9% (w/v) NaCl, and frozen overnight at -15°. The cells were disrupted by grinding in a cold mortar with twice their weight of alumina powder (Alcoa A-301). After grinding for 10 min., four times their weight of 0.1M-sodium phosphate buffer, pH 7.5, was added, and grinding continued for another 10 min. Homogenates were centrifuged at 8000 g for 10 min. in the cold, and the supernatant was used as 'crude extract'. The layer on the top of the alumina, removed and suspended in the above-named buffer, was used as 'large-particle fraction'.

Cofactors. Triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) were obtained from Sigma Chemical Co. The corresponding reduced nucleotides (TPNH and DPNH) were prepared enzymically (Nason & Evans, 1953; Pullman, Colowick & Kaplan, 1952). TPNH and DPNH concentrations were determined spectrophotometrically by using 6.22×10^6 cm.²/mole as extinction coefficient at 340 mμ (Horecker & Kornberg, 1948). Flavin-adenine dinucleotide (FAD) was obtained from Sigma Chemical Co.; flavin mononucleotide (FMN) from Nutritional Biochemicals Corp. and riboflavin from British Drug Houses Ltd. Boiled pig heart was prepared as described by Ochoa (1948). Vitamin K₃ (2-methyl-1:4-naphthaquinone bisulphite) was obtained from the Laboratorios Alter, dicoumarol from the Laboratorios Lefa, Madrid, Spain, and 2-heptyl-4-hydroxyquinoline-N-oxide was generously provided by Dr J. W. Cornforth, National Institute for Medical Research, Mill Hill, London.

Determination of enzymic activities. Nitrate-reductase activity was determined by measuring the nitrite formed with a modified sulphanilamide-naphthylamine test (Medina & Nicholas, 1957). Standard conditions were as follows: 0.1 ml. of enzyme was added at zero time to a mixture containing 0.1 ml. of 5 mM-DPNH, 0.1 ml. of 0.1M-KNO₃, 0.15 ml. of 0.1M-sodium phosphate buffer, pH 7.5, and vitamin K₃ when indicated, to a final volume of 0.6 ml., in open test tubes. After allowing the tubes to stand at 25° for 10 min., the reaction was stopped by adding M-barium acetate (0.1 ml.) and the volume made up to 5 ml. with 95% ethanol. After it was mixed, the suspension was centrifuged at 1500 g and tested for nitrite. To 1 ml. of the supernatant were added 1 ml. of sulphanilamide (1%, w/v, in 0.1M-HCl) and 1 ml. of α-naphthylamine hydrochloride (0.02%, w/v). After half an hour, colour intensity was read in a Klett-Summerson photoelectric colorimeter

* Deceased.

Table 1. *Effect of vitamin K₃ on nitrate-reductase activity*

Reaction mixtures containing 0.1 ml. of enzyme preparation (as described), 15 μ moles of sodium phosphate buffer, pH 7.5, 10 μ moles of KNO₃, 0.4 μ mole of DPNH and additions as indicated, in a final volume of 1 ml., were incubated at 25° for 10 min. Activity is expressed as μ m-moles of nitrite produced/10 min./mg. of protein. No nitrite was formed in a control without added DPNH.

Compound added	Synthetic medium		Complex broth medium	
	Crude extract (13.6 mg. of protein/ml.)	Large-particle fraction (33.8 mg. of protein/ml.)	Crude extract (6.9 mg. of protein/ml.)	Large-particle fraction (5.3 mg. of protein/ml.)
—	0	10	180	710
Boiled pig heart	0	10	190	700
FAD (0.1 μ mole)	0	10	190	700
Vitamin K ₃ (0.1 μ mole)	38	20	320	780
Atebrine (2 μ moles)	0	4	95	320
Atebrine (2 μ moles) + FAD (0.4 μ mole)	—	4	75	330
Atebrine (2 μ moles) + vitamin K ₃ (0.1 μ mole)	27	—	360	775
Atebrine (2 μ moles) + vitamin K ₃ (0.4 μ mole)	57	15	—	—
Dicoumarol (1 μ mole)	—	4	—	445
Dicoumarol (1 μ mole) + vitamin K ₃ (5 μ moles)	—	8	—	505

with the 500 μ filter. A control without added DPNH was always included. These aerobic conditions were used throughout unless specifically stated otherwise.

Menadione-reductase activity was determined spectrophotometrically, by following DPNH oxidation at 340 μ (Wosilait & Nason, 1954). Proteins were determined by the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

Activation of nitrate reduction by vitamin K₃. Extracts from *E. coli* grown in a synthetic medium or in a complex broth medium with nitrate were assayed for nitrate-reductase activity as described above. No nitrate-reductase activity was detected aerobically in crude extracts of *E. coli* grown in a synthetic medium, unless vitamin K₃ was added (Table 1). The system which was present in the crude extract and in the large-particle fraction was strongly stimulated by vitamin K₃. None of the flavin derivatives tested (FAD, FMN, and riboflavin) showed any activating effect nor did boiled pig heart. Dialysis overnight against 0.1 M-sodium phosphate buffer, pH 7.5, did not decrease the activity of the crude extract as long as the assay for nitrate reduction was carried out with the addition of vitamin K₃.

Atebrine inhibits the system, and the inhibition could not be reversed by FAD but was completely reversed by the addition of a similar amount of vitamin K₃.

The inhibition caused by dicoumarol was also reversed by vitamin K₃.

Aerobic oxidation of reduced diphosphopyridine nucleotide. Table 2 shows the oxidation of DPNH by a crude extract of *E. coli* grown in a synthetic medium. Activity was not affected by nitrate, but it was strongly increased by vitamin K₃. The latter

Table 2. *Aerobic oxidation of reduced diphosphopyridine nucleotide by a crude extract of Escherichia coli*

Mixtures containing 0.72 μ mole of DPNH, 0.1 ml. of enzyme (crude extract of *E. coli* grown in synthetic medium), 2.5 ml. of 0.1 M-sodium phosphate buffer, pH 7.5, and additions as indicated, in a final volume of 3 ml., were incubated for 10 min. at room temperature (18°). Oxidation of DPNH was followed spectrophotometrically at 340 μ . After 10 min. samples (1 ml.) were removed and placed in test tubes containing 0.1 ml. of M-barium acetate for determination of the nitrite formed, as described in the Materials and Methods section.

Additions	DPNH oxidized (μ m-moles)	Nitrite formed (μ m-moles)
None	50	0
KNO ₃ (100 μ moles)	50	0
Vitamin K ₃ (50 μ m-moles)	520	0
Vitamin K ₃ (50 μ m-moles) + KNO ₃ (100 μ moles)	675	225

result is explained by the presence in the extracts of menadione reductase (Wosilait & Nason, 1954). In the presence of both vitamin K₃ and nitrate, oxidation of DPNH was accompanied by nitrate reduction. This was not the case when vitamin K₃ was omitted. This suggests that vitamin K₃ can act as electron carrier between DPNH and nitrate.

If purified menadione reductase (Wosilait & Nason, 1954) was substituted for the above-mentioned extract, DPNH oxidation in the presence of vitamin K₃ was not accompanied by nitrate reduction. This observation demonstrates that nitrate reduction involves a different enzyme from the menadione reductase and was not an incidental by-product of the latter.

Inactivation by extraction with light petroleum, and reactivation by the extract and vitamin K₃. Crude

extracts and the 45P fraction (see below) from *E. coli* grown in a complex broth medium, which reduced nitrate even without the addition of vitamin K₃, lost a great deal of their nitrate-reductase activity by repeated extractions with

Table 3. *Inactivation of nitrate-reductase preparations by extraction with light petroleum and reactivation by the extract and vitamin K₃*

Nitrate-reductase activity was determined with 0.1 ml. of enzyme (*E. coli* grown in complex broth medium) by the standard procedure (see Materials and Methods section) with additions as indicated. Figures represent $\mu\text{m-moles}$ of nitrite formed in 10 min. at 25°. Dried material derived by extracting 20 ml. of boiled enzyme preparation with light petroleum (see text) was suspended in 1.5 ml. of water. In this experiment 0.2 ml. of this suspension was used.

Fractions	Additions		
	None	Light-petroleum extract	Vitamin K ₃ (1 μmole)
45 P	57.5	52.5	150
45 P extracted with light petroleum	10	37.5	152

Fig. 1. Effect of pH on nitrate-reductase activity. Mixtures containing 0.5 μmole of DPNH, 10 μmoles of KNO₃, 0.05 μmole of vitamin K₃, 0.3 ml. of 0.1 M-buffer as indicated, and 0.1 ml. of enzyme (45P fraction from *E. coli* grown in synthetic medium) in a total volume of 0.6 ml. were incubated for 10 min. at 25°. Nitrite was determined as in the standard procedure. The pH was determined in duplicate tubes. Buffers employed were: ●, sodium acetate; ○, sodium phosphate; ▼, glycine. All of these buffers were completely devoid of any inhibitory effect on the enzyme. Activity at pH 5.9 and 25° was 180 $\mu\text{m-moles}$ of nitrite formed/10 min.

light petroleum (b.p. 40–60°). Enzyme preparation was shaken in a test tube with an equal volume of light petroleum for 5 min. at room temperature and centrifuged. Residual nitrate reductase was determined by means of the standard procedure.

These preparations could be reactivated either by vitamin K₃ or by the dry residue obtained by vacuum evaporation of light-petroleum extracts of previously boiled preparations (see Table 3).

Solubility and stability of the system. Nitrate-reductase activity in crude extracts is about one-half of that in homogenates, which suggests that the system is of a particulate nature. This is supported by the fact that almost the whole activity was present in the sediment after centrifuging at 110 000 g for 1 hr. Treatment of the crude extracts with ammonium sulphate at 45 % saturation precipitates the system almost completely. This precipitate, suspended in 0.3 vol. of 0.1 M-sodium phosphate buffer, pH 7.5, is referred to as the 45P fraction.

Nitrate-reductase activity in the crude extracts was rather unstable. Upon storage at –20°, they lost about 75 % of their activity in 1 month. Under the same conditions the 45P fraction completely maintained its activity. This fraction was less stable after dialysis overnight against 0.1 M-phosphate buffer, pH 7.5.

The nitrate-reductase system completely lost its activity after 5 min. at 70° and pH 7.5.

Effect of pH upon nitrate-reductase activity. As shown in Fig. 1, activity was maximal in the range

Fig. 2. Effect of nitrate concentration on the rate of nitrate reduction. Mixtures containing 0.9 μmole of DPNH, 0.1 μmole of vitamin K₃, 15 μmoles of sodium phosphate buffer, pH 7.5, 0.1 ml. of enzyme (45P fraction from *E. coli* grown in synthetic medium) and nitrate as indicated, in a total volume of 0.65 ml., were incubated at 25° for 10 min. Nitrite formed was determined as in the standard procedure. ●, Reaction rate at various concentrations of nitrate; ○, double reciprocal plot (Lineweaver & Burk, 1934).

pH 6–7, with a particularly abrupt decrease below pH 6. Stability is maximal in the range pH 5–8, with a sharp drop above pH 9. Menadione-reductase activity has been reported to be maximal at pH 8.3 with a sharp drop above pH 9 (Wosilait & Nason, 1954).

Effect of concentration of nitrate and vitamin K₃ on the rate of nitrate reduction. Nitrate reduction by extracts of *E. coli* under standard conditions with an excess of vitamin K₃ was half maximal at about 0.1 mM-nitrate concentration (Fig. 2). With excess of nitrate, half-maximal activity was obtained at 0.05 mM-vitamin K₃, which is the order of magnitude of the *K_m* of the menadione reductase (Wosilait & Nason, 1954).

Effect of inhibitors upon the nitrate-reductase-menadione-reductase system. The strong inhibition produced by complexing agents such as cyanide, $\alpha\alpha'$ -dipyridyl and *o*-phenanthroline (Table 4) suggests the participation of some metal constituent in the system. The menadione reductase is not inhibited by complexing agents (Wosilait & Nason, 1954). Participation of a metal constituent in the process is supported by the inactivation produced by dialysis of the enzyme preparations against mM-cyanide.

The strong inhibition produced by 2-heptyl-4-hydroxyquinoline-*N*-oxide seems to take place at the vitamin K₃ level, since it could be reversed by increasing the vitamin K₃ concentration, in a manner suggesting competitive inhibition (Fig. 3).

Uncoupling reagents, such as 2:4-dinitrophenol, chlortetracycline and gramicidin, caused little inhibition. The system neither exhibited a phosphate requirement nor was it inhibited by pyrophosphate at concentrations of the order of 0.1 M.

Effect of nitrate on nitrate-reductase formation. Nitrate-reductase activity in crude extracts of *E. coli* grown in either synthetic or complex broth media without added nitrate was about 20 % of that in crude extracts from cells grown with nitrate, indicating that the nitrate reductase is inducible. Menadione-reductase activity was not dependent upon the presence of nitrate in the cultures.

Nitrate reduction in anaerobiosis. Crude extracts and the 45P fraction from *E. coli* grown in a synthetic medium did not reduce nitrate aerobically unless vitamin K₃ was added but reduced nitrate to nitrite under anaerobic conditions even without addition of vitamin K₃. This anaerobic reduction of nitrate was stimulated by FAD. The 45P fraction loses this capacity after 5 min. at 60°, whereas its stimulation by vitamin K₃ was resistant to this treatment (Table 5). Menadione-reductase activity was also present after this treatment. This observation is not in agreement with that reported by Wosilait & Nason (1954). Although the difference in results cannot be explained by the available data, it is suggested

Table 4. *Effect of inhibitors on the nitrate-reductase-menadione-reductase system*

Mixtures containing 0.1 ml. of crude extract (*E. coli* grown in synthetic medium), 10 μ moles of KNO₃, 15 μ moles of sodium phosphate buffer, pH 7.5, 0.5 μ mole of vitamin K₃, 0.5 μ mole of DPNH and 0.1 ml. of inhibitor (to give final concentrations as indicated) were incubated in a final volume of 1 ml. Enzyme and inhibitor had been mixed and preincubated for 10 min. at 25° before the addition of the assay reagents. After a further incubation of 10 min., nitrite formed was determined as in the standard procedure.

Compound	Concentration (mM)	Inhibition (%)
KCN	1	100
$\alpha\alpha'$ -Dipyridyl	6	67
<i>o</i> -Phenanthroline	5	90
Sodium diethyldithiocarbamate	1	29
Salicylic acid	1	32
8-Hydroxyquinoline	1	29
Thiourea	1	41
2-Heptyl-4-hydroxyquinoline- <i>N</i> -oxide	0.4	95
Urethane	10	0
2:4-Dinitrophenol	0.1	18
Chlortetracycline	0.05	19
Gramicidin	0.02	19

Fig. 3. Competitive inhibition of vitamin K₃ by 2-heptyl-4-hydroxyquinoline *N*-oxide. Representation of Lineweaver & Burk (1934). Mixtures containing 0.5 μ mole of DPNH, 10 μ moles of KNO₃, 15 μ moles of sodium phosphate buffer, pH 7.5, 0.1 ml. of enzyme (crude extract of *E. coli* grown in synthetic medium) and vitamin K₃ and inhibitor (*I*) as indicated, in a total volume of 1 ml., were incubated for 10 min. at 25°. Nitrite formed was determined as in the standard procedure. ●, Inhibitor present; ○, inhibitor absent.

Table 5. *Effect of vitamin K₃ and flavinadenine dinucleotide upon the aerobic and anaerobic reduction of nitrate*

Mixtures containing 10 μ moles of KNO₃, 1.3 μ mole of DPNH, 15 μ moles of sodium phosphate buffer, pH 7.5, and 0.2 ml. of enzyme (*E. coli* grown in synthetic medium) were incubated for 10 min. at 25°, in a final volume of 0.9 ml. Assays under anaerobic conditions were made in Thunberg tubes. Additions of 0.002 μ mole of FAD or 0.2 μ mole of vitamin K₃ were made as indicated. Activity is expressed as μ m-moles of nitrite formed/mg. of protein.

Fraction	Anaerobic				Aerobic			
	None	FAD	Vitamin K ₃	FAD + vitamin K ₃	None	FAD	Vitamin K ₃	FAD + vitamin K ₃
Ppt. from ammonium sulphate at 0-45% saturation	14	35	420	470	0	0	390	390
Ppt. from ammonium sulphate at 0-45% saturation heated for 5 min. at 60°	0	0	320	310	0	0	280	280

that the heat lability found by Wosilait & Nason could be related to the acetone treatment involved in their preparation.

DISCUSSION

Reduction of nitrate to nitrite in *E. coli* seems to take place through pathways which differ in some of the electron carriers and are detectable depending on the assay conditions. Aerobically, the process is strongly stimulated by addition of vitamin K₃. The presence of a pyridine nucleotide-dependent menadione reductase (Wosilait & Nason, 1954) suggests that, *in vivo*, vitamin K₃ or some related compound acts as electron carrier between DPNH or TPNH and nitrate. This hypothesis is supported by the fact that vitamin K₃ can act at catalytic levels, which suggests that vitamin K₃ is oxidized and reduced alternately. Further support for this contention is provided by the observation that chemically-reduced vitamin K₃ can act as electron donor for the nitrate reductase (A. Medina, unpublished work). The possibility that nitrate could be reduced by reduced vitamin K₃ without participation of a second enzyme is ruled out by the fact that purified menadione reductase is unable to reduce nitrate even in the presence of vitamin K₃.

The strong inhibition produced by complexing agents suggests that some metal is involved in the process. The fact that menadione-reductase activity is not inhibited by these compounds indicates that the metal is involved in the electron transfer from reduced vitamin K₃ to nitrate. The inhibition produced by 2-heptyl-4-hydroxyquinoline-*N*-oxide, which is supposed to be an inhibitor in processes where cytochrome *b*₁ is involved (Lightbown & Jackson, 1954), seems to be competitive with vitamin K₃.

Crude extracts from *E. coli* grown in a complex

broth medium, which reduce nitrate even without any addition of vitamin K₃, can be inactivated by extraction with light petroleum. Reactivation of the residue, by either vitamin K₃ or the ether extract, suggests that the role of vitamin K₃ *in vitro* is accomplished *in vivo* by some lipo-soluble factor, possibly vitamin K₂, which is known to occur in *E. coli* (Baumgärtel & Zahn, 1953).

The aerobic reduction of nitrate was not stimulated by flavins. On the other hand, atebriene, generally considered as typical inhibitor of flavoenzymes, was inhibitory. Nevertheless, the fact that the inhibition by atebriene could be completely reversed by vitamin K₃ (but not by FAD) suggests that no flavoenzyme is involved (Haas, 1944). Nicholas & Nason (1955) have demonstrated the occurrence of protein-bound FAD in a preparation of nitrate reductase from *E. coli*. Nevertheless, the fact that they report an activity in anaerobiosis over twice that in air suggests that their preparation was enriched in DPNH oxidase, which could account for the FAD found (see below).

Anaerobically, nitrate reduction can be stimulated not only by vitamin K₃ but also by FAD. The activity stimulated by FAD can be destroyed by heat-treatment without major impairment of activity stimulated by vitamin K₃. These facts suggest that there are at least two mechanisms through which nitrate can be reduced to nitrite. In one of them, detectable under aerobic as well as in anaerobic conditions, vitamin K₃ or some related compound acts as electron carrier, and, in another one, detectable only under anaerobic conditions, flavin is involved. This fact is in agreement with the finding of Taniguchi *et al.* (1956), who propose that anaerobic nitrate reduction via flavin takes place through an electron-transport chain with some of the electron carriers in common with the DPNH-oxidase chain. Nitrate with terminal nitrate reductase can serve as the terminal

Scheme 1

respiratory acceptor in anaerobiosis, instead of oxygen plus terminal oxidase, which is the normal acceptor in anaerobiosis.

Taniguchi *et al.* (1957) were unable to obtain any stimulating effect by vitamin K_3 on DPNH oxidation. This was presumably due to a loss of the menadione-reductase activity in their preparations. Since the completion of this work, Itagaki & Taniguchi (1959) have reported activation of extracts by vitamin K_3 , thus confirming our preliminary report that the latter can be involved in nitrate reduction (Medina & Heredia, 1958).

Our results suggest that the scheme proposed by Taniguchi *et al.* (1957) should be modified to include an alternative pathway which can work in aerobiosis as well as in anaerobiosis. Only the identified steps are included in the minimal Scheme 1.

Although the possibility that the last stage in both processes, the electron transfer from an electron carrier to nitrate, could be catalysed by two different enzymes cannot be excluded, it seems more likely, from specificity considerations, that it is catalysed by a single enzyme. The specific thermal inactivation of the system in which flavin is involved does not necessarily support the participation of two different enzymes, since inactivation could take place in earlier steps of the chain.

The fact that nitrate reduction via flavin seems to take place only under anaerobic conditions suggests that it is intimately linked to the energy metabolism of the cell. In this case nitrate would act as an alternative terminal oxidant, according to the 'nitrate-respiration' theory (Sato, 1950). On the other hand, the fact that the nitrate-reductase system involving vitamin K_3 is oxygen-insensitive and the known ability of *E. coli* to grow on nitrate as the only source of nitrogen, suggests that this system can play a role in nitrogen assimilation. This contention is supported by the observation of the ability of intact *E. coli* to reduce nitrite and hydroxylamine (Taniguchi *et al.* 1956) and the identification in extracts of nitrite- and hydroxyl-

amine-reductase activities (A. Medina, unpublished work).

SUMMARY

1. A system which reduces nitrate to nitrite, with di- or tri-phosphopyridine nucleotide as electron donor and vitamin K_3 as electron carrier, has been found in *Escherichia coli*.

2. The transfer of electrons from pyridine nucleotides to vitamin K_3 is catalysed by the constitutive menadione reductase. Nitrate reductase proper is an inducible enzyme which is associated with particulate matter. Nitrate reductase apparently involves some metal but not flavin.

3. The effects of substrate concentration, pH and other factors have been investigated.

4. Anaerobically, in addition to this pathway, nitrate reduction can take place through a system, apparently flavin-dependent, presumably related to the reduced diphosphopyridine nucleotide oxidase.

5. The possible physiological significance of these two systems able to reduce nitrate is discussed.

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The Effect of Growth and Function on the Chemical Composition of Soft Tissues

BY ELSIE M. WIDDOWSON AND J. W. T. DICKERSON

Medical Research Council, Department of Experimental Medicine, University of Cambridge

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During the second half of the nineteenth century a considerable amount of work was done in European countries on the chemical composition of the mammalian body and of its component tissues. The subject then lapsed, and papers on it appeared only occasionally until 10 or 15 years ago, when there was a revival of interest, particularly in the United States. Much of the work has been done on animals, but human tissues have also been studied, generally to see how various diseased states affected their composition.

In spite of all this activity there have, up till now, been very few systematic studies of the changes which take place in tissues with normal development. It has been known for at least 100 years that foetuses and new-born animals contain a higher proportion of water than do adults of the same species (von Bezold, 1857). This is to some extent accounted for by an increasing proportion of fat in the body, but even on a fat-free basis the percentage of water in the whole body falls from embryonic to some time in post-natal life. This percentage then becomes approximately constant at an age which varies from species to species. This 'steady state' was termed by Moulton (1923) 'chemical maturity'. More recently it has been appreciated that the fall in the percentage of water is due largely to a decrease in the proportion of the body occupied by extracellular fluids. Coincident with this there is a rise in the proportion of the body occupied by cells, the cells first increasing primarily in number and later in size.

The body is made up of many different organs and tissues, each with a different chemical composition, and each contributing a different proportion to the body as a whole. The proportion which the various organs contribute to the body, moreover, is not the same at all ages; for example, the brain always accounts for a larger proportion of the body in early life, and skeletal muscle accounts for more of the weight of the adult than of the foetus or new-born baby. The present investigation was designed to study, in man and the pig, the rate at which different parts of the body become chemically mature, and if possible to relate this to the function of these parts at different ages. The effect of development on the chemical composition of the whole bodies of these two species was described some years ago (Widdowson & Spray, 1951; Widdowson, McCance & Spray, 1951; Spray & Widdowson, 1951). More recently the findings on the serum and erythrocytes have been published (Widdowson & McCance, 1956; McCance & Widdowson, 1956a) and also those on the skeletal muscle (Dickerson & Widdowson, 1960).

MATERIAL AND METHODS

The sources of the material for this investigation were the same as those for the skeletal muscle described by Dickerson & Widdowson (1960). Skin, heart, liver, kidney and brain have been studied at four or five stages of development in man and five or six stages of development in the pig. The number of samples analysed in each case is given in the tables. Each sample from the foetal and new-born