5. Whereas the cholinesterase activity remains approximately constant, the tributyrin activity increases somewhat, and the methyl butyrate activity increases markedly with age.

6. It is concluded that the cholinesterase of skin is a true, specific cholinesterase.

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Bacterial Tetrathionase: Adaptation without Demonstrable Cell Growth

(A Report to the Medical Research Council)

By R. KNOX AND M. R. POLLOCK, *Emergency Public Health Laboratory, Leicester*

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In suitable conditions micro-organisms react to changes in their chemical environment by changes in enzymic activity. Two types of process may be distinguished. In the first, the new property develops slowly in successive cultures and, once developed, remains stable even in the absence of the specific substrate. In the second, the new property appears after a delay of only a few hours when the cells are brought into contact under appropriate conditions with the specific substrate, but is rapidly lost when they are subcultured in an environment not containing it (Dubos, 1940). Yudkin (1932) suggested that the term 'training' be used for the first process, and 'adaptation' for the second. 'Training' may well be brought about by natural selection. 'Adaptation' is almost certainly not, since the time for development of the new property is too short for appreciable cell division to have occurred. With the formic hydrogenlyase of *Esch. coli*, Stephenson & Stickland (1932) showed that adaptation could occur without evidence of cell division but only in an environment which was favourable for maximal growth, since they had to add full strength nutrient broth to their suspensions in order to produce adaptation. Stephenson &

Yudkin (1936), however, found with the galactozymase of *Saccharomyces cerevisiae* that adaptation could occur slowly in the absence of added nitrogen source and without any significant cell division, while Hegarty (1939) found that washed suspensions of *Streptococcus lactis* could be adapted to ferment galactose in the absence of either nitrogen source or cell division. Pollock, Knox & Gell (1942) and Pollock & Knox (1943) showed that washed suspensions of *Bact. paratyphosum* B prepared from agar cultures could be adapted to reduce sodium tetrathionate rapidly to thiosulphate in accordance with the equation

simply by preliminary treatment for 2–3 hr. at 37° with tetrathionate and a suitable hydrogen donor. The present paper gives in detail the evidence that this adaptation occurs without detectable growth.

METHODS

Several strains of *Bact. paratyphosum* B were used; some were freshly isolated from clinical cases, others were stock laboratory cultures maintained on egg slopes. One ml. of an 18 hr. broth culture was inoculated on to the surface of bottles containing 30–40 ml. of nutrient agar prepared with

tryptic digest broth; after allowing the inoculum to run over the whole surface the bottles were incubated for 15–18 hr. at 37°. The growth was then washed off with 5–6 ml. of $\frac{1}{4}$ strength Ringer's solution (abbreviated: ' $\frac{1}{4}$ Ringer') per bottle, the washings were centrifuged and the deposit resuspended in 2 ml. of ' $\frac{1}{4}$ Ringer' solution for each bottle. A solution containing phosphate buffer (pH 7.6), phenol red, iodide-free sodium tetrathionate and mannitol was prepared and warmed to 37°. On addition of the bacterial suspension, separately warmed to 37°, the suspension mixture contained 0.2M phosphate buffer, 0.002% phenol red in ethanol (giving a final concentration of 1% ethanol in the suspension mixture), 0.02M tetrathionate, 0.02M mannitol and bacterial suspension in a dilution of 1 in 5. Reduction of tetrathionate at 37° was recorded by removal of samples at intervals during the reaction and titration with 0.005N- I_2 (Pollock & Knox, 1943). When all the tetrathionate had been reduced the remainder of the suspension mixture was centrifuged and the deposit resuspended to give about the same final concentration of bacteria in a solution of the same composition as before. Reduction of tetrathionate at 37° was again observed by the same technique.

Total and viable counts were made on the suspension mixture at the beginning and end of the adaptation process.

Total counts. A 1 mm. Helber counting chamber (depth 0.02 mm.) with a 1 mm. dark ground condenser and $\times 25$ compensating eyepiece was used with a 16 mm. objective (Wilson, 1922). A convenient dilution for counting was 1/500 of the suspension mixture (in 1% phenol). Duplicate series of dilutions were made for all counts, and except in Exps. 11 and 12, duplicate counts were made on each dilution. At least two rows of 20 squares (i.e. 2×10^{-6} ml.) were counted in each estimation. A rough estimate of the average bacterial size was made at the time of counting. For the **viable counts**, duplicate series of dilutions were made in ' $\frac{1}{4}$ Ringer' and the number of colonies developing from a suitable dilution on the surface of agar plates was counted. The counts in all cases were done in duplicate and in two experiments comparison with the standard pour-plate method showed good agreement. **Dry weights** were estimated by taking 5 ml. samples, centrifuging, washing with distilled water and drying at 100–105° to constant weight. In the last two experiments nephelometric comparison of the suspension mixture at the beginning and end of the adaptation process was made by Dr Elkins, using a photo-electric nephelometer.

RESULTS

A typical experiment is illustrated in Fig. 1. Reduction of tetrathionate by a suspension of organisms from nutrient agar in the presence of mannitol proceeded at a rapidly increasing rate (curve A) until, after about 2 hr., the rate became linear. The same suspension (centrifuged after adaptation and resuspended in buffer-mannitol-tetrathionate solution) showed linear reduction from the start at a rate approximately equal to that of the original suspension at the end of the adaptation process (curve A'). This linear rate in the second half of the experiment is in fact evidence that the adaptation process was complete by the end of the first half. With unadapted suspensions the initial rate

of tetrathionate reduction is too low for accurate measurement; there may or may not be a genuine lag period before any increase in rate begins. In the middle part of the curve the iodine values (indicating the amount of tetrathionate reduced to

Fig. 1. Tetrathionate reduction by *Bact. paratyphosum* B during and after adaptation. Suspension mixture contained 0.02M tetrathionate, 0.2M phosphate, and 0.02M mannitol. (Complete bacterial reduction of 1 ml. of 0.02M tetrathionate to thiosulphate gives a titration value of 8 ml. of 0.005N- I_2 .)

thiosulphate) increase at an approximately logarithmic rate, until the linear phase is reached when adaptation appears to be complete. Table 1 gives the results of 12 consecutive experiments designed to show whether any increase in bacterial protoplasm occurs during the adaptation process. It can be seen that there is no evidence of any increase in either the total or viable cell counts; if anything, there is a suggestion of a slight fall in the viable count during the course of the experiments. The dry weights show no significant increase, though the possibility of a slight increase in weight during adaptation cannot be dismissed. No difference in bacterial size before and after adaptation was noticed in any of the experiments. In the last two experiments duplicate 1/500 dilutions of the suspension mixtures were examined nephelometrically: the readings were identical at the beginning and the end of the adaptation process. Several strains of *Bact. paratyphosum* B were used in these experiments.

'Conway' was a stock strain which had been subcultured several times at long intervals on egg slopes; 1838 was a recently isolated strain from a case of paratyphoid B fever;

Table 1. *Reduction of sodium tetrathionate by suspensions of Bact. paratyphosum B*

Exp. no.	Strain	Remarks	Per ml. suspension mixture*						Rate of tetra-thionate reduction	Dura-tion of exp. (min.)
			Beginning			End				
			Total count $\times 10^9$	Viable count $\times 10^9$	Dry wt. (mg.)	Total count $\times 10^9$	Viable count $\times 10^9$	Dry wt. (mg.)		
1	Conway	Kept 1 day at 4°	~	~	3.3	~	~	3.2	4.8	150
2	Conway	Kept 3 weeks at 4°	19.4	16.2	3.1	17.5	13.4	3.1	1.65	180
3	Conway	—	13.3	11.4	~	12.8	9.93	~	4.4	180
4	1838	Kept 3 weeks at 4°	35.0	10.35	~	35.0	10.7	~	4.6	180
5	1838	Freshly prepared	16.0†	18.0	~	21.2	17.4	~	9.2	97
6	1838	Same suspension as 5. Kept 5 days at 4°	20.6	17.5	~	20.3	13.7	~	4.2	150
7	Murdoch 1	Kept 2 yr. on egg, used fresh	23.2	16.3	~	21.6	12.3	~	2.0	210
8	Murdoch 2	Freshly isolated, used fresh	21.0	20.5	~	20.5	16.0	~	2.0	180
9	3135	Freshly isolated, used fresh	26.15	18.9	~	24.0	14.7	~	3.2	180
10	3332	Isolated 5. viii. 43. Kept 1 day at 4°	33.7	25.6	4.5	33.6	21.65	4.6	8.0	165
11	3332	Isolated 5. viii. 43. Kept 1 day at 4°	48.75	41.1	8.6	48.6	40.0	8.72	7.0	165
12	266	Freshly isolated. Kept 1 day at 4°	24.75	19.1	4.4	24.0	15.1	4.78	7.0	165

* Total and viable counts are expressed as number of cells $\times 10^9$ /ml. suspension mixture, dry weights as mg./ml. suspension mixture. Rates of tetrathionate reduction are expressed as iodine values (in terms of 0.005N-I₂) given by 1 ml. samples of the suspension mixture. (Complete reduction of 1 ml. of 0.02M tetrathionate gives a reading of 8 ml. of 0.005N-I₂.) Rate of tetrathionate reduction at beginning of experiment was always low, and usually about 0.2.

† Result presumably inaccurate.

'Murdoch 1' was originally isolated (2 years previously), during the acute stage of her illness, from a woman who subsequently became a chronic carrier; 'Murdoch 2' was freshly isolated from the same patient 2 years later; 3135 was freshly isolated from a case; and 3332 and 266 were freshly isolated from two carriers remaining from two different outbreaks of paratyphoid B fever.

The interval between the preparation of a suspension and its use in an experiment was not always the same (see Exps. 5 and 6). It can be seen that the ratio between the concentration of bacterial suspension and the final rate of tetrathionate reduction varied widely in different experiments. Once fully adapted in this way these suspensions retained their power of rapid and linear reduction of tetrathionate for long periods. Repeated washing, though it slightly decreased the actual rate of tetrathionate reduction, never caused any deviation from a linear reaction. Further, these suspensions, if kept at 4° for several weeks, still gave a linear reaction though the rate slowly diminished with time of storage. The same suspensions, however, rapidly lost the newly acquired property of linear reduction on sub-culture and growth in a tetrathionate-free medium.

Conditions for maximal adaptation. In all the experiments of which details are given in Table 1 and Fig. 1 'adaptation' curves similar to curve A in Fig. 1 were obtained. This may therefore be regarded provisionally as the 'normal' or typical curve, at least for the particular conditions of these

experiments. Additional experiments showed that certain alterations in the composition of the sus-

Fig. 2. Effect of different mannitol concentrations during adaptation. Suspension mixtures as in Fig. 1, but with mannitol in concentrations from 0.04 to 0.001M.

pending fluid produced deviations in shape and velocity from the 'normal' curve. Different concen-

trations of the constituents of the complete suspending fluid were tested. (1) *Phenol red* in the concentration used (0.001 %) had a definite delaying

Fig. 3. Effect of different phosphate concentrations during adaptation. Suspension mixtures as in Fig. 1, but with phosphate in concentrations from 0.4 to 0.02M, and also with phosphate omitted.

effect on the reaction. This was due to the ethanol with which the indicator was made up. The final concentration of ethanol in the suspension mixture was

1 %, and 1 % ethanol alone without phenol red had exactly the same inhibitory effect. (2) *Mannitol*. Identical curves were obtained between 0.04 and 0.005M; below 0.005M the curve was atypical (Fig. 2). Mannitol was used rather than glucose, which gave even more rapid curves but from which *Bact. paratyphosum* B produces acid more rapidly, making the pH difficult to control. (3) *Phosphate*. The optimum concentration appeared to be nearer 0.1 than 0.2M (Fig. 3). With lower concentrations, although the reaction was accelerated in the early stages, the rate fell off rapidly because of the low pH developed as a result of reduction of tetrathionate to thiosulphate (Pollock & Knox, 1943). In the experiments given in Table 1 and Fig. 1, however, 0.2M was used rather than 0.1M phosphate in spite of the somewhat slower reaction, because the pH did not fall appreciably until near the end. (4) *Tetrathionate*. 0.02M was found in practice to be sufficient for maximal adaptation.

The part played by the phosphate, tetrathionate and mannitol separately and together was investigated. Fig. 4 illustrates a typical experiment. It consisted of two parts.

(1) A bacterial suspension prepared from a culture grown on nutrient agar was treated in five different ways, by incubation at 37° for 3 hr. in the presence of (A) phosphate, tetrathionate and mannitol, (B) phosphate and tetrathionate, (C) phosphate and mannitol, (D) phosphate and (E) '¼ Ringer'. Samples from the tubes containing tetrathionate (A and B) were titrated with 0.005N-I₂ (curves A and B). The contents of all five tubes were then brought to pH 7.6, kept at 4° overnight and centrifuged. (2) The resuspended deposits were then all incubated alike with

Fig. 4. Treatment of bacterial suspension in different ways. (a) During treatment: for explanation see text. (b) After treatment.

phosphate, tetrathionate and mannitol and samples titrated with 0.005 N- I_2 in the usual way (curves A' , B' , C' , D' , E'). Complete and rapid adaptation occurred only when both tetrathionate and mannitol were present during treatment (A). Incubation with ' $\frac{1}{4}$ Ringer' alone, followed by centrifuging, greatly delayed the adaptation process (E'); so did incubation with phosphate alone, though to a less degree (D'). Incubation with mannitol and phosphate produced a suspension (C') which then adapted itself more rapidly than a suspension incubated with ' $\frac{1}{4}$ Ringer' or phosphate, but much more slowly than the original suspension incubated with tetrathionate and mannitol (A). A suspension incubated with tetrathionate and phosphate alone showed during that treatment a slow adaptation (B) and after treatment a further slow adaptation (B') with a rate which was still not linear after 160 min., whereas with the fully adapted suspension reduction of tetrathionate was rapid and linear from the start (A).

DISCUSSION

The total and viable counts show that during adaptation no appreciable cell multiplication occurs. It is, of course, possible that any multiplication which did occur would not be detectable because of actual disappearance of cells by lysis, but it is unlikely that multiplication and lysis would occur at exactly the same rate. It is also possible that the power to reduce tetrathionate is restricted to a very small proportion of the cells initially present, that these cells alone are capable of multiplying in the conditions of our experiments, and that the great increase in rate of tetrathionate reduction is due entirely to multiplication of those particular cells. Neither of these possibilities could be completely eliminated by the methods we have used, nor perhaps by any technique that might be devised. It is also possible to criticize the use of bacterial counts as evidence of cell growth, since in cultures in which growth is known to be occurring it is common to find cells which have divided (often more than once) but still remain joined in one unit. These units would be counted as single cells by any method of counting. Further, during the lag phase of bacterial growth, great cell enlargement is known to occur. Neither enlargement nor incomplete cell division, if they occurred during adaptation, could be detected by counts alone. Estimations of dry weight, nephelometric comparisons and direct observations of cell size, taken together with the total and viable counts, strongly suggest, however, that adaptation is not accompanied by any significant increase in either number or size of cells, and therefore depends on factors other than a simple increase in bacterial protoplasm.

Because of the rapidity of the process, its occurrence without demonstrable cell multiplication, and

the rapid loss of the new property on subculture, it is evident that natural selection is not involved and that there is true adaptation in the sense used by Yudkin. There are many ways in which adaptation might occur—by synthesis of enzyme or coenzyme, progressive removal of some inhibitor, alteration in cell membrane permeability, and other physico-chemical changes on the surface or inside the cell. Without further data any attempt to explain the mechanism involved would be speculative. It would even be unwise to assume at this stage that the deviations from the 'normal' curve observed when alterations are made in the composition of the suspending fluid represent genuine effects on the adaptation process itself. The facts at present available show, however, (1) that rapid adaptation can occur without demonstrable increase in bacterial protoplasm and without any added nitrogen source and (2) that the combined presence of the specific hydrogen acceptor (tetrathionate) and a suitable hydrogen donator (mannitol) is necessary to produce complete and rapid adaptation. This supports Dubos's suggestion that for maximal adaptation the mere presence of the substrate is not sufficient; it must also be actively metabolized.

SUMMARY

1. Washed suspensions of *Bact. paratyphosum* B, prepared from cultures grown on nutrient agar, when incubated at 37° with tetrathionate, phosphate and mannitol, show at first a limited power of reducing tetrathionate to thiosulphate.
2. After an initial period of $\frac{1}{4}$ –1 hr. a rapid and apparently logarithmic increase in rate of reduction occurs until a linear phase is reached, when adaptation appears to be complete.
3. This increase in enzymic activity occurs in the absence of added nitrogen source, and without demonstrable increase in cell numbers or cell material.
4. Cells which have developed this property, when centrifuged and resuspended in tetrathionate, phosphate and mannitol, show rapid and linear reduction of tetrathionate from the start.
5. The new property is maintained after repeated washing of the cells and storage at 4° for several weeks.
6. Optimal development of the new property seems to require the combined presence of the specific hydrogen acceptor (tetrathionate) and a suitable hydrogen donator.

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Chromatographic Investigation of the β -carotene Content of Serum of the Newborn Infant

By H. HOCH (Freedom Research Scholar), *Hale Clinical Laboratory, London Hospital*

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Several workers have shown that the carotenoid level of the blood from the umbilical cord of newborn infants is considerably lower than that of the mother (Menken, 1934; Gaetgens, 1937; Willstaedt & With, 1939; Byrn & Eastman, 1943; Hoch, 1943 a; Lewis, Bodansky & Shapiro, 1943). On the other hand, the last three of these investigators found that the vitamin A levels are of the same order in both. However, since the serum carotenoids are a mixture of similar substances, it seemed of interest to investigate the concentration of the biologically active β -carotene in the serum of mother and baby.

EXPERIMENTAL

Collection of blood samples. Venous blood was taken from healthy women repeatedly before and after parturition. At parturition, blood was collected from the cord and from the vagina and the samples left to clot in the dark. In a few instances venous blood was oxalated and the plasma used. Contamination of vaginal samples by other fluids was avoided as far as possible; diluted samples were, however, included in this set of analyses, since no serious error was to be expected in the relative proportions of carotenoids. This error and also that due to possible hydraemia may affect the values for the concentration of total carotenoids. In several instances the protein concentration was determined to obtain an estimate of the dilution of the blood.

Method of extraction of carotenoids from serum. 1 ml. of maternal and 1–4 ml. of foetal serum were used. 1.00 ml. of serum (or plasma) was placed in a 15 ml. narrow-necked centrifuge tube and 0.15 ml. of 29% NaOH, 0.5 ml. ethanol and a few drops of light petroleum (boiling range 40–60°) were added in succession with thorough mixing after each addition. About 3 ml. light petroleum and 0.65 ml. ethanol were then added and the mixture shaken up immediately to avoid precipitation of protein, which occurs if an equal volume of ethanol is added directly to serum. Cork stoppers wetted with distilled water were used and the mixtures were shaken by hand for 5 min. The extraction was repeated twice with about 1.5 ml. light petroleum. The pooled extracts were washed three times with distilled water, transferred

to a dry centrifuge tube and, after addition of 0.3–0.4 g. anhydrous Na_2SO_4 , evaporated to dryness in a stream of N_2 (bath temperature 50–55°). The extract was then dissolved in *n*-hexane and transferred quantitatively to a colorimeter tube, made up to exactly 1 ml. and centrifuged (2600 r.p.m.) until completely clear.

Determination of carotenoid content of extract. The extinction of the hexane solution was measured photoelectrically, with an Ilford Spectrum violet filter no. 601. The values of the extinction were obtained by subtracting the $\log_{10}$ (galvanometer reading for the unknown) from the $\log_{10}$ (galvanometer reading for the control (*n*-hexane)). The light intensity was adjusted so as to give a reading for the control between 95 and 105 mm. on the scale. The calibration was carried out with recrystallized chromatographically homogeneous β -carotene. Solutions of this were made up in *n*-hexane. An extinction of 0.1 corresponded to 0.62 μg . β -carotene. Correction was made for the non-linearity of the galvanometer. The deviation from proportionality of concentration and extinction was found to be less than 1% in the range normally used (extinction < 0.45) and greater than 2% only with extinctions above 0.8.

A photoelectric colorimeter was built on the principle of the Evelyn instrument (Evelyn, 1936) and its essential details are given here in so far as they differ from those of the Evelyn model. The light source was a 6 V. 6 c.p. car side-light bulb fed from a car accumulator (60 amp. hr.). The light intensity was varied by means of a series stud resistance. Each step altered the galvanometer readings by about 5%. The maximum current taken was 0.57 amp. No reflector was used. The colorimeter tubes were matched ordinary centrifuge tubes 90 $\times$ 9.5 mm. with an internal diameter of 8 mm. The light beam was limited by a stop to illuminate an area of 10 $\times$ 2 mm. on the colorimeter tube. The distance from the filament to the centre of the tube was 30 mm. A Calorex glass (infra-red filter) of 1.5 mm. thickness (manufactured by Chance Bros. Ltd.) was mounted close to the bulb and the light filters could be placed immediately in front of the photoelectric cell. The latter was an 'EEL' selenium barrier layer cell (manufactured by Evans Electro-selenium Ltd.). The galvanometer used (sensitivity 840 mm./ μa ., 2 sec. periodic time and 175 Ω coil resistance) is a standard type S.S. 6/45 of H. Tinsley and Co.