Short Communications

Inhibition of Citrate Metabolism by Sodium Fluoroacetate in the Perfused Rat Heart and the Effect on Phosphofructokinase Activity and Glucose Utilization

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Recently three groups of workers (Parmeggiani & Bowman, 1963; Passonneau & Lowry, 1963; Garland, Randle & Newsholme, 1963) have reported that citrate is a potent inhibitor of phosphofructokinase, and it has been shown further in certain conditions associated with a physiological inhibition of phosphofructokinase that intracellular citrate concentration is elevated (Parmeggiani & Bowman, 1963; Garland *et al.* 1963). Attempts in this Laboratory to demonstrate an inhibition of phosphofructokinase in rat heart perfused with citrate have failed, apparently owing to the fact that citrate is not removed from the perfusion medium by the heart. An alternative approach to elevating intracellular citrate concentration is through the use of sodium fluoroacetate. Peters and his colleagues (Liébecq & Peters, 1949; Buffa & Peters, 1949; Peters, Wakelin, Buffa & Thomas, 1953; Morrison & Peters, 1954) have shown that fluoroacetate is converted intracellularly into fluorocitrate and that this latter compound competes successfully for aconitase. The result is the development of very high concentrations of intracellular citrate, as well as hyperglycaemia and ketonaemia (Engel, Hewson & Cole, 1954). Cole, Engel & Fredericks (1955) observed that there was a relatively good correlation between this 'fluoroacetate diabetes' and tissue citrate concentrations. The present experiments were carried out to determine if the rise in citrate concentration induced by fluoroacetate is accompanied by an inhibition of phosphofructokinase.

Methods. Hearts from rats fed *ad libitum* were removed while the animals were anaesthetized with sodium pentobarbital, and were perfused with or without sodium fluoroacetate (obtained from Sigma Chemical Co.) by the recirculation technique described by Morgan, Henderson, Regen & Park (1961). Apart from a slight increase in the flow rate of the perfusion medium, no effect of fluoroacetate was noted on the mechanical behaviour of the hearts. At the end of a 20 min. perfusion period, the hearts were frozen between blocks of aluminium cooled in liquid nitrogen, and pulverized in a stainless-steel mortar at liquid nitrogen temperature. The frozen powder was then extracted in either 10 % (w/v) trichloroacetic acid or 10 % (v/v) perchloric acid. Samples of the trichloroacetic acid extracts were assayed for citrate by the pentabromoacetone method of Natelson, Pincus & Lugovoy (1948). Neutralized perchloric acid extracts were assayed enzymically for hexose phosphates and ATP. Enzymes for analyses were obtained from Boehringer und Soehne G.m.b.H. The methods employed were those described by Hohorst (1963) for glucose 6-phosphate and fructose 6-phosphate, by Bücher & Hohorst (1963) for fructose 1,6-diphosphate and by Lamprecht & Trautschold (1963) for ATP. A Gilford recording spectrophotometer was used for measuring extinction changes. Glucose uptake and intracellular glucose concentrations were determined according to techniques given by Morgan *et al.* (1961).

Results. The results given in Table 1 show that the concentration of citrate in the heart was greatly increased by perfusion with sodium fluoroacetate. Phosphofructokinase activity was judged by the equilibrium that developed between glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate. In control hearts perfused without fluoroacetate the fructose 6-phosphate concentration was approximately twice that of fructose 1,6-diphosphate. In hearts perfused with 0·22 mM-fluoroacetate the fructose 6-phosphate concentration was 16 times that of fructose 1,6-diphosphate. This change in ratio was due to a threefold increment in the fructose 6-phosphate concentration and a 60 % fall in that of fructose 1,6-diphosphate. The glucose 6-phosphate concentration increased to approximately the same extent as that of fructose 6-phosphate. Associated with this apparent block in phosphofructokinase activity there was a decrease in glucose uptake and an increase in the intracellular concentration of free glucose, indicating an inhibition of glucose phosphorylation. Only a small decrease in ATP concentration was found. In hearts perfused with a lower concentration of fluoroacetate (0·076 mM)

Table 1. *Effect of sodium fluoroacetate on the concentrations of citrate, ATP and hexose phosphates and on hexokinase activity in the perfused rat heart*

The perfusion medium was composed of Krebs–Henseleit bicarbonate buffer, pH 7·4, containing glucose (5·6 mM) and insulin (100 milliunits/ml.). Sodium fluoroacetate was present in the perfusion medium (*in vitro*) at the concentrations indicated, or was injected intraperitoneally into the animals (*in vivo*) 2 hr. before the removal of the hearts for perfusion; in the latter case no fluoroacetate was present in the perfusion medium. Glucose phosphorylation is taken to be the glucose disappearance from the perfusion medium minus the accumulated intracellular glucose at the end of the perfusion period. The results are given as means ± S.E.M. n.m., Not measured.

Conditions	No. of hearts/ group	Concn. of citrate (μmoles/g. dry wt.)	Concn. of ATP (μmoles/g. dry wt.)	Concn. of glucose 6-phosphate (μmoles/g. dry wt.)	Concn. of fructose 6-phosphate (μmoles/g. dry wt.)	Concn. of fructose 1,6-diphosphate (μmoles/g. dry wt.)	Intracellular concn. of glucose (mM)	Glucose phosphorylation (μmoles/min./ g. dry wt.)
Control	12	2·0 ± 0·1	13·2 ± 0·6	1·5 ± 0·04	0·32 ± 0·01	0·17 ± 0·02	0·59 ± 0·22	4·67 ± 0·15
Fluoroacetate (0·22 mM) *in vitro*	12	20·9 ± 0·3	10·7 ± 0·8	4·2 ± 0·2	0·99 ± 0·04	0·06 ± 0·01	3·5 ± 0·3	2·1 ± 0·1
Control	8	2·4 ± 0·4	n.m.	1·5 ± 0·09	0·35 ± 0·04	0·19 ± 0·02	1·15 ± 0·44	5·73 ± 0·2
Fluoroacetate (0·076 mM) *in vitro*	7	13·2 ± 1·5	n.m.	2·65 ± 0·13	0·56 ± 0·04	0·09 ± 0·02	2·66 ± 0·3	4·5 ± 0·15
Control	12	2·2 ± 0·1	15·0 ± 0·8	1·5 ± 0·04	0·26 ± 0·05	0·18 ± 0·03	1·15 ± 0·14	4·94 ± 0·2
Fluoroacetate (30 μmoles/kg.) *in vivo*	12	5·5 ± 0·7	6·9 ± 0·3	1·4 ± 0·04	0·31 ± 0·06	0·06 ± 0·02	4·18 ± 0·46	0·69 ± 0·2

citrate also accumulated and phosphofructokinase inhibition was indicated by the changes in hexose phosphate concentrations. A smaller accumulation of intracellular glucose developed than with the higher fluoroacetate concentration, but there was still a small decrease in glucose uptake, reflecting a decreased rate of glucose phosphorylation.

In another experiment rats were injected with fluoroacetate (30 μmoles/kg.) 2 hr. before the removal of the hearts for perfusion and no fluoroacetate was included in the perfusate. Before the perfusion, hearts from animals injected with fluoroacetate had very high citrate concentrations (approx. 23 μmoles/g. dry wt.), but after a 20 min. perfusion period the concentration had declined considerably (Table 1). Estimation of hexose phosphates in these hearts after perfusion showed, in comparison with perfused hearts from non-treated animals, a decrease in fructose 1,6-diphosphate concentration, little or no increase in fructose 6-phosphate concentration and no increase in glucose 6-phosphate concentration. In spite of this marginally apparent inhibition of phosphofructokinase and a normal concentration of glucose 6-phosphate, there was an accumulation of intracellular glucose and a severe inhibition of glucose uptake, indicating a very low rate of glucose phosphorylation. The concentration of ATP was decreased by half in perfused hearts from fluoroacetate-injected rats.

Discussion. These experiments support the hypothesis that intracellular citrate may be a regulator of phosphofructokinase. Hearts perfused directly with fluoroacetate or hearts from animals pretreated with fluoroacetate had elevated citrate concentrations and a correlated inhibition of phosphofructokinase activity. Inhibited glucose phosphorylation and inhibited glucose uptake were also observed. These last-named effects may have been due, in part, to the inhibition of phosphofructokinase and resultant accumulation of glucose 6-phosphate, since this intermediate is an inhibitor of hexokinase (Crane & Sols, 1955). However, in hearts from animals injected with fluoroacetate before perfusion, the results suggest that other factors also may have been involved. Although the concentration of fructose 1,6-diphosphate decreased, fructose 6-phosphate and glucose 6-phosphate concentrations failed to increase. It appears that there was an inhibition of hexokinase that approximated the inhibition of phosphofructokinase. Engel *et al.* (1954) postulated that the hyperglycaemia observed in rats injected with fluoroacetate might be due to a decreased glucose phosphorylation rate. They suggested that a deficiency of ATP consequent to fluoroacetate blockage of the citric acid cycle might account for depressed hexokinase activity. The present results

do show that the concentration of ATP was considerably decreased in hearts from animals treated with fluoroacetate, but it is not known whether these depressed ATP concentrations are below that required for adequate hexokinase activity in the heart.

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Abnormal Sulphatase Activities in Two Human Diseases (Metachromatic Leucodystrophy and Gargoylism)

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Recent evidence suggests that lysosomal enzymes may play an important role in the balance between health and disease (de Reuck & Cameron, 1963). In the present controlled study, lysosomal enzyme activities were determined in fresh frozen human brains, kidneys, livers and urines. Characteristic changes in sulphatases A and B were found in two genetically-determined human 'storage' diseases. The sulphatase changes are of interest for two reasons: first, in their general biological relationship to the many puzzling aspects of sulphate ester metabolism, and, secondly, in their potentially more specific relationship to the excess of sulphated molecules found in each disease.

One disease, metachromatic leucodystrophy, is characterized chiefly by an increase in sulphatides (cerebroside sulphates) in the devastated myelin of the nervous system and in the kidney (Austin, 1960, 1964). In the second disorder, gargoylism, the increase is chiefly in sulphated mucopolysaccharides (chondroitin sulphate B and heparitin sulphate) (Meyer, Hoffman & Linker, 1957; Dorfman & Lorincz, 1957).

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Tissue samples were obtained at autopsy from four metachromatic-leucodystrophic children (aged 6–12 years); urine samples were studied in nine living children with the same disease. Samples were also taken from two autopsied gargoyle children aged 4 and 12 years; urine samples were studied in five such children still living. Pertinent abnormal autopsy control material included three children with globoid (Krabbe) leucodystrophy (aged 23 months–5 years) (Austin, 1964). Five other 'normal' autopsy controls were comparable with the pathological material both in age of patient and in duration of storage at $-30°$. Relevant controls in the study of urine included other family members as well as patients with various storage diseases (Niemann-Pick disease and glycogenosis). All autopsy and urine samples were analysed simultaneously in large controlled series.

Thawed tissues were homogenized for 2 min. with distilled deionized water at the medium-high-speed setting in a VirTis blender. This and subsequent operations were performed at 4°. Homogenates were centrifuged for 30 min. at 10000 rev./min. (12000g) in a Servall centrifuge.

The first sediment was frozen, thawed and resuspended. Enzyme assays were done on the clear combined supernatants obtained after constant exchange dialysis against distilled water in presoaked Visking tubing for 14 hr. Urines were uninfected first morning samples. After centrifuging or filtration through glass wool the supernatants were thoroughly dialysed and were finally concentrated by polyethylene glycol, pervaporation or freeze-drying.

Assay methods included: arylsulphatase type A and type B (Baum, Dodgson & Spencer, 1959), acid phosphatase (Sigma Chemical Co., 1963) and, in organs only, β-galactosidase (modification of the method of Conchie & Hay, 1963). Enzyme activities were related to mg. of total soluble protein (Lowry, Rosebrough, Farr & Randall, 1951) to give the activity/protein ratios.

The most obvious result was the low arylsulphatase-A activities in all organs from metachromatic-leucodystrophic children. In four such brains the mean arylsulphatase-A activity was only 0·9. These samples included either grey matter or white matter or both and had specific activities in the range 0–2·0. In three kidneys the mean arylsulphatase-A activity was only 0·8 (range 0·3–1·4). Arylsulphatase-A activity was not detectable in any of three livers. In control brains, by contrast, the mean arylsulphatase-A activity was 27·3 (range 7·2–59·3; $n=10$), in nine control kidneys it was 30·5 (range 11·2–99·2) and in nine control livers it was 43·1 (range 5·5–91·0). Two samples of brain cortex from metachromatic-leucodystrophic children could be clearly separated by dissection from white matter. In these two cases (twins with the classical form of metachromatic leucodystrophy) it was clear that the arylsulphatase-A activity was low in both cortex and in white matter. For example, in the white matter it averaged only 1·6 (1·1 and 2·0); by contrast, in four control samples of white matter it averaged 42·2 (range 26·1–59·3). In the cortex the mean arylsulphatase-A activity was only 0·7 (0·4 and 1·0), whereas in two control samples it was 20·2 (13·5 and 26·8). Descriptively, the metachromatic-leucodystrophic patients appear to provide the first documented evidence of hyposulphatasia in man.

The changes in arylsulphatase-B activity differed from those in arylsulphatase-A activity. Only two metachromatic-leucodystrophic patients had consistently low arylsulphatase-B activities. These patients were siblings (not twins) who had the unique variation of the disease in which cortical grey matter is involved and sulphated polysaccharides are increased (Austin, 1964). For example, arylsulphatase-B activity was not detectable in either of their brain samples. These samples were a mixture of approx. 70 % of cortical grey matter and 30 % of white matter. In controls the activity averaged 16·4 (range 9·3–30·3) in four comparably mixed brain samples. In three kidneys the mean arylsulphatase-B activity was 2·3 (range 0–5·1) and in three livers it was 2·6 (range 2·3–2·9). In each instance the low activity was from the variant form of metachromatic leucodystrophy. By contrast, the mean arylsulphatase-B activity in nine control livers was 8·2 (range 2·0–24·8) and in nine control kidneys it was 21·8 (range 5·2–45·8).

In the twins with classical metachromatic leucodystrophy cited above, arylsulphatase-B activity was low only in the devastated white matter (2·5 and 2·8). In three non-gargoyle control samples of white matter the mean arylsulphatase-B activity was 4·9 (range 2·7–7·5). However, in the essentially intact cortex of these metachromatic-leucodystrophic twins, the arylsulphatase-B activity was not obviously diminished: the values obtained were 23·2 and 26·3; that of one normal control cortex was 10·6.

In marked contrast with the arylsulphatases, acid-phosphatase specific activities in tissues from metachromatic-leucodystrophic children were not appreciably different from those in controls. In brains, for example, the mean acid-phosphatase activity was 2·4 (range 1·2–4·0), that in controls being 2·0 (range 0·6–3·5). In kidneys the mean acid-phosphatase activity was 3·1 (range 2·9–3·5), that in controls being 4·0 (0–10·0). In livers the mean acid-phosphatase activity was 2·5 (range 1·4–3·3), that in controls being 2·8 (range 0·3–4·2).

β-Galactosidase activities in tissues from metachromatic-leucodystrophic children were not appreciably different from those in controls. In brains the mean β-galactosidase activity was 3·9 (range 2·4–5·3), that in controls being 3·6 (range 0·8–6·2). In kidneys the mean β-galactosidase activity was 2·8 (range 1·2–4·3), that in controls being 3·0 (range 1·4–4·4). In livers the mean β-galactosidase activity was 2·4 (range 0·6–3·6), that in controls being 3·0 (range 0·8–5·4).

The changes in arylsulphatase activities in gargoyle children were different from those in metachromatic-leucodystrophic children. In gargoyle children the mean arylsulphatase-B activity in liver was 23·7; (range 22·5–24·8). Moreover, samples from gargoyle children had the highest arylsulphatase-B activities in white matter (17·1) and in cortex (33·2) and the highest mean activity (30·5) in kidney (15·3 and 45·8). By contrast, all three other lysosomal enzymes were either in the normal or low-normal range in tissues from gargoyle children.

The above-mentioned changes in arylsulphatase activities were also observed in metachromatic-leucodystrophic children during life and hence were not post-mortem artifacts. Urine was

selected for study because arylsulphatases produced in the kidney normally enter the urine. The mean arylsulphatase-A activity in urines from metachromatic-leucodystrophic children was only 1·1: five of the nine children had no detectable arylsulphatase-A activity on one or more occasions. Urine arylsulphatase-A activity in the other four children was always low (range 0·3–4·3). The 4·3 value was exceptional because previous values with this patient were 0·0 and 0·0, and a subsequent one was only 0·2. By contrast, the mean arylsulphatase-A activity in control urine was 52·2 in children and 56·5 in adults (the overall range was 0·6–447·3 in 123 normal and abnormal control samples). The low control value (0·6) was from a patient with nephrosis, and it demonstrates that a gross excess of albumin may easily diminish a specific activity ratio. Normal parents and siblings of metachromatic-leucodystrophic children usually had values within the normal range with one exception (one sibling aged 9 years: not detectable on one occasion; 2·2, in the low-normal range, on one other test). A carrier state in this child manifested by a low arylsulphatase-A activity during immaturity might be one possible explanation for these findings. With the approach used, the range of arylsulphatase-B activity in urine has been wide. No significant differences were noted between metachromatic-leucodystrophic children (mean 10·3; range 0–89·4), gargoyle children (mean 6·8; range 0–2·60) and normal children (mean 8·1; range 0–133·7).

All four enzymes studied have been localized to lysosomes. Hence the finding in metachromatic-leucodystrophic patients of essentially normal values for (lysosomal) acid-phosphatase and β-galactosidase activities is of some note. These findings would make it difficult to attribute the low sulphatase activities to a simple secondary non-specific degeneration of lysosomes in metachromatic leucodystrophy. Nor, in separate experiments, do inhibitors explain the low arylsulphatase activities. The present findings suggest that it is chiefly arylsulphatase-A activity that is universally low in metachromatic leucodystrophy and that it is arylsulphatase-B activity that is increased in gargoylism. These findings are a more specific extension of previous findings in which an undifferentiated lowering of arylsulphatase activity was noted in metachromatic leucodystrophy and an undifferentiated increase in arylsulphatase activity was noted in gargoylism (Austin *et al.* 1963). The precise relationship between these changes in arylsulphatase activities and the turnover defect of sulphuric acid esters in metachromatic leucodystrophy and gargoylism has not yet been established.

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The Effect of Ethionine on the Synthesis of Nicotinamide–Adenine Dinucleotide and Nicotinamide–Adenine Dinucleotide Phosphate in Rat Liver

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Among the factors that could affect the rate of synthesis of the nicotinamide nucleotides in tissues are the activities of the enzymes involved in the synthetic pathways and the availability of substrates for these enzymes. The failure to demonstrate any correlation between the activities of

NMN adenylyltransferase (EC 2.7.7.1) and of NAD^+ kinase (EC 2.7.1.23), measured under optimum conditions, and the concentrations of NAD and NADP in either pre-cancerous livers (Clark, Greenbaum & McLean, 1964) or the livers of rats subjected to a variety of hormonal treatments (Greenbaum, Clark & McLean, 1965*a*), suggest that substrates may play an important regulatory role in these synthetic processes. The injection of tryptophan, nicotinic acid or, in particular, nicotinamide into rats leads to a very marked increase of hepatic NAD^+, but to a much smaller increase of NADP (Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach, 1956; Greengard, Quinn & Reid, 1964), suggesting that the synthesis of NAD is a substrate-limited process. The synthesis of NADP from NAD is a single-step process involving one enzyme, NAD^+ kinase, and two substrates, NAD^+ and ATP. Since the synthesis of NADP *in vivo* does not appear to be much stimulated by high endogenous concentrations of NAD^+ (up to ten times normal concentrations) and neither does it appear to be correlated with variations in the maximum potential activity of the enzyme measured *in vitro*, it seemed possible that the concentration of ATP *in vivo* could be an important regulator of NADP synthesis.

A convenient method for studying the effect of changes in the concentration of ATP *in vivo* on the synthesis of nicotinamide nucleotides was suggested by the work of Shull (1962) and of Farber, Shull, Villa-Trevino, Lombardi & Thomas (1964), who found that the injection of ethionine into female rats lowered the concentration of ATP in the liver by as much as 80%. In the experiments reported below the activities of NMN adenylyltransferase and NAD^+ kinase were measured, as were the concentrations of the nicotinamide nucleotides in the livers of normal female rats and of similar rats that had been injected with 500 mg. of L-ethionine/kg. body wt. intraperitoneally 5 hr. before being killed. This time was chosen because Shull (1962) found the maximum effect on ATP concentrations after this time-interval. The assay systems employed were as follows. NMN adenylyltransferase was measured in whole homogenates prepared in 0·25M-sucrose essentially as described by Kornberg (1950). NAD^+ kinase was measured in the soluble fraction obtained by centrifuging 0·15M-KCl–0·16 mM-$KHCO_3$ homogenates for 135000*g*-min. essentially as described by Wang & Kaplan (1954). The NAD^+, NADH, $NADP^+$ and NADPH were extracted in either 0·1N-HCl or 0·1N-NaOH and determined separately as described by Greenbaum, Clark & McLean (1965*b*) by using an enzymic recycling method with either alcohol dehydrogenase (EC 1.1.1.1) or glucose 6-phosphate dehydrogenase (EC 1.1.1.49). In Fig. 1 the results are shown as the combined NAD^+ + NADH and $NADP^+$ + NADPH since it is the total amount of nicotinamide nucleotide with which these experiments are concerned. Nevertheless, treatment with ethionine caused no marked shifts in the relative amounts of the oxidized and reduced forms of the nucleotides.

Though there is no change in the liver content of NAD or of the enzyme catalysing its formation from NMN, there is a considerable fall in the concentration of NADP with no associated change in the activity of the enzyme that synthesizes it from NAD. The lack of effect of ethionine on the NMN adenylyltransferase is in accordance with the results of Stirpe & Aldridge (1961).

It may be suggested on the basis of these results that the effects of ethionine *in vivo* in causing a fall in NADP may be related to the known action of this agent in decreasing the hepatic ATP concentration. What is, perhaps, surprising in the light of this suggestion is that it is NADP that is affected and not NAD, in spite of the fact that ATP is required for the synthesis of both nucleotides. Whether this difference arises from the different intracellular localizations of the two

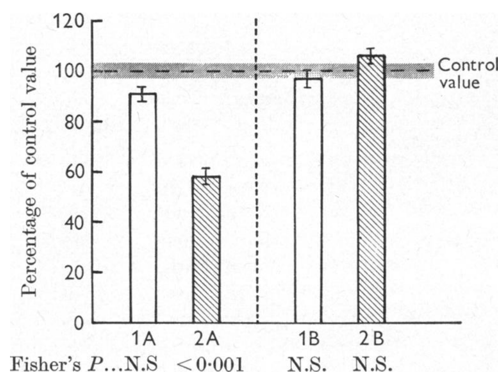

Fig. 1. Effect of ethionine on the content of NAD and NADP and the activities of NMN adenylyltransferase and NAD^+ kinase of rat liver. The rats were treated with 100 mg. of L-ethionine 5 hr. before being killed. The oxidized and reduced forms of the nicotinamide nucleotides were extracted in 0·1N-HCl or 0·1N-NaOH respectively and all four nucleotides estimated separately. Column 1A, NAD^+ + NADH; column 1B, $NADP^+$ + NADPH. NMN adenylyltransferase (column 1B) was estimated in 0·25M-sucrose homogenates by a modification of the method of Kornberg (1950). NAD^+ kinase (column 2B) was estimated in the supernatant obtained by centrifuging a liver homogenate in 0·15M-KCl–0·16 mM-$KHCO_3$ for 135000*g*-min. by a modification of the method of Wang & Kaplan (1954). The results are expressed as a percentage of the control values. The vertical bars represent twice the S.E.M. Fisher's *P* values (six rats in each group) are shown when the value is less than 0·1; N.S., not statistically significant.

enzymes and a changed distribution of ATP as a result of ethionine action or whether it is a reflexion of the relative K_m values of the two reactions is not entirely clear. But a study of the ATP requirements of NMN adenylyltransferase and NAD^+ kinase by measurements of the apparent K_m values in studies *in vitro* reveals an interesting difference between the two enzymes: the former enzyme has K_m 0·12 mM (Morton, 1961), but the NAD^+ kinase of pigeon-liver preparation appears to have a much higher K_m, approx. 18 mM (calculated from the data of Wang & Kaplan, 1954). In the present studies with rat-liver preparations it was found that similar high concentrations of ATP are required for this enzyme. Thus it appears that the present results may be accounted for, in part, by this large difference of over 100-fold in the K_m values. It is possible that NADP concentrations in various hormonal conditions will also be found to be related to the available ATP.

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Unusual Polysomal Aggregates in Rat-Liver Preparations

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There have been many reports describing aggregates of ribosomes said to be held together by strands of messenger RNA and which are active in protein biosynthesis (Rich, Warner & Goodman, 1963). The work of Wettstein and his colleagues on ribosomal aggregates in rat liver suggests that there is a broad distribution of polyribosomal sizes containing from 2 to 25 or more single ribosomes. Most aggregates contained 5, 6 or 7 ribosomes, and these are said to be most active in protein synthesis (Wettstein, Staehelin & Noll, 1963). To what extent these are fragments resulting from the breakdown of much larger aggregates during the disruption of the cells, particularly in techniques involving mechanical shearing forces, is uncertain. In the present report the presence of large polysomal aggregates in rat-liver preparations is described. The effects of homogenizing techniques and other factors on these aggregates are also reported.

Methods. Male white rats of the Wistar strain were raised under specific pathogen-free conditions. Animals weighing 200–300 g. were killed by a blow on the head and bled freely. The livers were chilled, minced with scissors and homogenized in 2·5 vol. of a medium containing 0·04M-tris–HCl buffer, pH 7·7 (40 mM), KCl (25 mM), $MgCl_2$ (4 mM) and sucrose (0·25M) by using a Perspex pestle in a stainless-steel tube (clearance 1 mm.) at 6 strokes at 1000 rev./min. After centrifuging for 15 min. at 15000g to remove mitochondria and debris, the supernatant was treated with $\frac{1}{9}$ vol. of 11% (w/v) sodium deoxycholate.

The supernatant (0·1 ml.) was immediately layered in the form of an inverse gradient (total vol. 0·2 ml.) over a linear 10–25% (w/v) sucrose gradient containing tris–HCl buffer, pH 7·4 (10 mM), KCl (25 mM) and $MgCl_2$ (4 mM). The tube was centrifuged in the SW 39 rotor of the Spinco model L 50 centrifuge for a total of 45 min. at 39000 rev./min. Samples (42) were collected by puncturing the tube and were diluted fivefold before measurement of the extinction at 260 mμ. Samples were examined in the electron microscope by applying them directly to carbon-coated grids and washing in buffer containing decreasing concentrations of sucrose. The grids were air-dried and shadowed with tungsten at an angle of 5:1.

Results. Fig. 1 shows three components moving faster than 120s ribosomes and having S values of approx. 225, 275 and 340s. The protein/RNA ratio of the material in these peaks was very nearly 1:1.

Under the conditions described above this pattern was extremely reproducible and was unaffected by prior starvation of the rats for 18 hr. Layering the deoxycholate-treated supernatant directly on the gradient rather than as an inverse gradient did not affect the positions of the peaks but lowered the resolution. Treatment of the supernatant with 10 μg. of ribonuclease/ml. for 5 min. at 37° before the addition of deoxycholate converted the three fast-moving components into a mixture of 120 s and 80 s ribosomes.

With Mg^{2+} concentrations between 1·5 and 10 mM in both homogenizing medium and gradient patterns similar to that shown in Fig. 1 were obtained; the use of 0·1 mM-Mg^{2+}, however, resulted in the breakdown of the fast-moving components and an increase in the material at the top of the gradient. Omission of K^+ from the homogenizing medium and gradient also degraded the three fast-moving components. The degree of severity of the homogenization of the liver had a profound effect on the $E_{260\,m\mu}$ profile: 12 strokes with the loose-fitting pestle or 6 strokes with a tight-fitting pestle (clearance 0·125 mm.) gave a more polydisperse pattern and an increase in the material at the top of the gradient in the latter case.

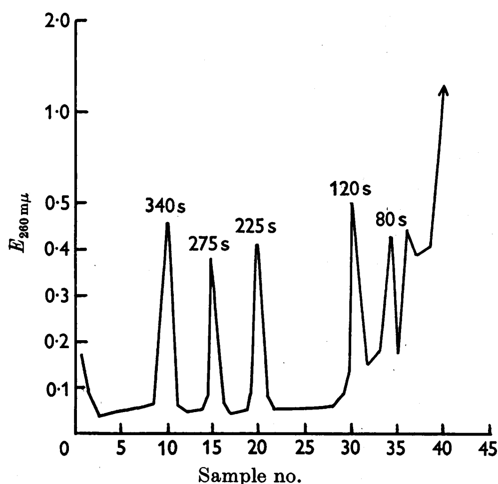

Fig. 1. Sedimentation pattern of deoxycholate-treated 15000*g* supernatant of rat-liver homogenate. A 0·1 ml. sample was layered as an inverse gradient (total vol. 0·2 ml.) over a linear 10–25% (w/v) sucrose gradient, and centrifuged in the SW 39 rotor for 45 min. at 39000 rev./min. at 0°. Approximate sedimentation coefficients of the peaks are indicated. Full details are given in text.

Finally, the concentration of the deoxycholate used to solubilize the endoplasmic reticulum was found to be important: a final deoxycholate concentration of 0·5% in the 15000*g* supernatant lowered the resolution of the peaks a little but the basic pattern was unchanged; at 1·3% (w/v) the pattern was identical with the control; but at 1·66% (w/v) all the peaks were completely degraded. Omission of deoxycholate produced an entirely different pattern (of poor reproducibility) from the control.

Preliminary electron micrographs have indicated ribosomal aggregates of decreasing size in the 340 s, 275 s and 225 s fractions. Accurate counting of the number of ribosomes in the clusters was not possible, but in the 340 s material there appeared to be between 30 and 40 ribosomes. The ribosomes in the 120 s fraction were dimers and monomers.

Discussion. The results suggest the presence of polysomal aggregates in the discrete peaks moving faster than dimerized ribosomes. They are fragile and a more polydisperse distribution is produced by more severe homogenization. The effects of very low Mg^{2+} and K^+ concentrations and the treatment of the 15000*g* supernatant with ribonuclease suggest that the aggregates are stabilized by RNA, Mg^{2+} and K^+. Further, the degradative effect of increasing the deoxycholate concentration to 1·66% suggests that the aggregates may be stabilized by a membrane, although the presence of phospholipid has yet to be demonstrated. The work of Hardesty, Arlinghaus, Schaeffer & Schweet (1963) suggests that polysomes, at least in reticulocytes, are stable in 0·1 mM-Mg^{2+}, so that the three fast-moving components in 4 mM-Mg^{2+} reported above might be regarded tentatively as Mg^{2+}-bound aggregates of smaller polysomes since they were degraded in 0·1 mM-Mg^{2+}. The significance of the striking reproducibility and resolution of these aggregates under the standard conditions described is uncertain. It has not yet been possible to demonstrate their presence in homogenates of livers of rats of the same strain not raised under specific pathogen-free conditions.

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Determination of Succinic Acid by an Enzymic Method

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Several enzymic methods have been recommended for the determination of succinate. These include manometric assays with washed heart or skeletal muscle preparations (Weil-Malherbe, 1937; Krebs, 1937) and spectrophotometric methods based on: (i) the reduction of cytochrome *c* in the presence of a trace amount of phenazine methosulphate (Massey, 1959); (ii) the reduction of cytochrome *c* in the presence of cyanide (Bach & Hibbitt, 1959); (iii) the reduction of ferricyanide (Singer, Bernath & Lusty, 1963); (iv) the reduction of 2,6-dichlorophenol-indophenol (Vishniac & Ochoa, 1952; Waitzman & Adams, 1958; Rodgers, 1961); and (v) the reduction of 2,3,5-triphenyltetrazolium chloride (Bril, 1954).

Manometric methods have the advantage that a preliminary extraction of succinic acid from cell homogenates is not necessary, and a relatively large number of determinations can be performed at one time. On the other hand, they are not generally as sensitive nor as rapid as spectrophotometric methods. However, some spectrophotometric methods have limited applicability to homogenates and other turbid suspensions (Waitzman & Adams, 1958; Massey, 1959; Rodgers, 1961; Singer *et al.* 1963) and may require the use of highly purified enzyme preparations (Massey, 1959; Singer *et al.* 1963).

The method described below has the following advantages over other spectrophotometric methods: (i) the enzyme is more easily prepared than that used by Massey (1959) or Singer *et al.* (1963); (ii) the sensitivity is greater than for the methods of Bril (1954), Bach & Hibbitt (1959) and Singer *et al.* (1963); (iii) the number of determinations that can be carried out at one time is not limited as in methods where the reaction is carried out in the cuvettes of a spectrophotometer (Vishniac & Ochoa, 1952; Waitzman & Adams, 1958; Massey, 1959; Bach & Hibbitt, 1959; Singer *et al.* 1963); (iv) it allows the determination of succinic acid in turbid solutions or flocculent suspensions; (v) the quantity of succinic acid is proportional to an increase rather than to a decrease in extinction, as in some other methods (Vishniac & Ochoa, 1952; Waitzman & Adams, 1958; Rodgers, 1961; Singer *et al.* 1963).

A similar method to the one described below is that of Bril (1954), in which the formazan produced by the reduction of 2,3,5-triphenyltetrazolium chloride is estimated colorimetrically. However, the sensitivity of the method (approx. 0·1–1·5 μmoles) is less than that of the present method. This method, which is suitable for the determination of 0·01–0·2 μmole of succinic acid, depends on the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-tetrazolium chloride in the presence of an excess of succinate dehydrogenase. The resulting formazan is extracted into ethyl acetate and its extinction measured. The method was developed from an assay for succinate-dehydrogenase activity described by Pennington (1961) (see also Shelton & Rice, 1957).

A crude succinate-dehydrogenase preparation was obtained as follows. Fresh rat liver was homogenized for 1 min. in 5 vol. of cold 0·25 M-sucrose with a loose-fitting Teflon-tipped homogenizer and centrifuged at 1000 **g** for 10 min. to remove nuclei and cell debris. A mitochondrial sediment was obtained from the supernatant by centrifuging at 10 000 **g** for 15 min. The sediment was resuspended in 10 vol. of 0·25 M-sucrose and dialysed overnight at 4° against glass-distilled water initially adjusted to pH 7 with $NaHCO_3$.

The assay procedure is as follows. Samples (0·2 ml.) of a 0·5 % solution of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-tetrazolium chloride (British Drug Houses Ltd., laboratory reagent) are added to centrifuge tubes each containing 0·5 ml. of a mixture of phosphate buffer, pH 7·6 (50 μmoles), EDTA (2·5 μmoles) and sucrose (25 μmoles). The tubes are placed in ice and dialysed rat-liver mitochondrial preparation is added (0·1 ml. of a suspension containing about 5–10 mg. of protein/ml.), followed by the unknown or standard succinate solutions to give a final volume of 1 ml. in each tube. The pH of the final reaction mixture is 7·6. The tubes are kept in ice for a further 15 min. and then incubated at 37° for 60 min. (or longer if necessary, depending on the activity of the preparation). The reaction is terminated by the

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addition of 1 ml. of 10 % (w/v) trichloroacetic acid. The tubes are cooled in ice, ethyl acetate (4 ml.) is added and the contents of each tube are vigorously shaken. After a brief centrifugation the extinctions of the upper ethyl acetate layers are measured in a 1 cm. cuvette at 490 mμ.

The lower and upper limits of the method are 0·01 and 0·2 μmole respectively, and within this range there is a linear relationship between $E_{490\,m\mu}$ and the quantity of succinate. The mean value for $E_{490\,m\mu}$ obtained with 0·1 μmole of succinate was 0·45 (S.D. ± 0·01) (six determinations) for one enzyme preparation. The slope of the line varied with different mitochondrial preparations but was usually linear. Extinction values of 0·31, 0·32, 0·42 and 0·45 were obtained for 0·1 μmole of succinate added initially with four different enzyme suspensions. The production of formazan as a function of time with a constant amount of succinate (0·1 μmole) is shown in Fig. 1. The reaction proceeded in this experiment to within 86 % of the theoretical value. Values as high as 92 % and as low as 62 % have also been obtained. These percentages are calculated from a molar extinction coefficient for the formazan dissolved in ethyl acetate of $2{\cdot}01 \times 10^4$ (Pennington, 1961). To establish the point at which the reaction is completed a preliminary time curve should be carried out with each new enzyme preparation or when the mitochondrial suspension has been stored for some time.

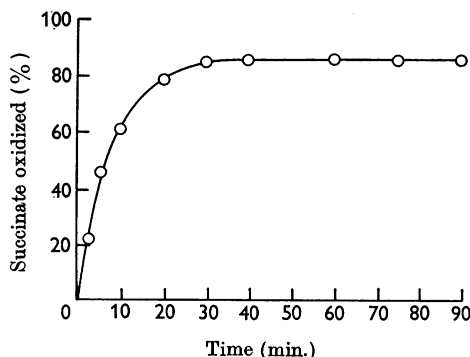

Fig. 1. Time curve for the estimation of succinic acid. Incubation at 37° was carried out for different times with a constant amount of succinic acid (0·1 μmole). The reaction was terminated, and the formazan extracted and measured at 490 mμ as described in the text. The points shown are the means of duplicate determinations.

Several compounds (10 μmoles of each) reacted in the assay system, in the absence of added succinate, to give extinction values equivalent to the following amounts of succinate: sodium acetate, 0; sodium L-glutamate, 0·01 μmole; sodium oxaloacetate, 0·02 μmole; sodium citrate, 0·02 μmole; sodium fumarate, 0·03 μmole; and sodium α-oxoglutarate, 0·04 μmole. When 10 μmoles of sodium acetate, sodium citrate, sodium pyruvate, sodium α-oxoglutarate or sodium L-glutamate were added to assay tubes containing 0·1 μmole of succinate, the following increases in the extinction values were observed: 1, 3, 11, 11 and 16 % respectively. Sodium fumarate (10 μmoles) decreased the yield of formazan from 0·1 μmole of succinate by 30 %, sodium malonate (10 μmoles) by 84 %, and sodium oxaloacetate (10 μmoles) by 85 %.

The small effects of large excesses of acetate, L-glutamate, pyruvate and citrate, and the marked inhibitions by malonate and oxaloacetate, agree with the results of Rodgers (1961), who used a different method for estimating succinic acid. However, in contrast with results obtained by Rodgers (1961), we have found that high concentrations of fumarate interfere in the assay whereas high concentrations of α-oxoglutarate have little effect. Bril (1954), using a similar method to the one described above, found no interference in the assay by citrate, pyruvate or α-oxoglutarate but inhibition by high concentrations of fumarate.

If large amounts of fumarate, malonate, oxaloacetate or other inhibitors are expected or known to be present, suitable precautions, such as those described by Rodgers (1961), should be taken.

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