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Specific Metabolic Repression of Three Induced Enzymes in *Escherichia coli*

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It is well known that the synthesis of many induced catabolic enzymes in micro-organisms is repressed by glucose. Earlier studies on β -galactosidase formation in *Escherichia coli* have established the following facts (see Mandelstam, 1961, 1962). The glucose effect is not specific, and any compound that the cells can use as a source of carbon and energy can repress the formation of β -galactosidase, although it may not do so in usual conditions of growth, i.e. in a shaken flask culture. Any carbon-source metabolite will cause repression when the bacteria are starved of nitrogen or magnesium or a specific auxotrophic requirement, and the supply of carbon from the substrate concerned is in excess of what can be used for growth. The degree of repression produced by a compound is directly correlated with its effectiveness as a growth substrate. These results are consistent with the assumption that it is not glucose itself, but some metabolite derived from it, that is the active repressor (see Neidhardt & Magasanik, 1956; Mandelstam, 1961).

It has also been shown that the repressor that produces the glucose effect does so both in constitutive and induced synthesis of enzyme, and the effect is not reversed by inducer added even in large amounts (Mandelstam, 1962). It therefore appears to be entirely distinct from the repressor that has been postulated by Jacob & Monod (1961) as the cause of inducibility. For this reason we shall refer to it in this paper as the metabolic repressor. The term 'catabolite repression' has been used to describe the effect (Magasanik, 1961). Since the present experiments and previous work (Mandelstam, 1962) show that the repressor can be derived not only catabolically but also anabolically from

relatively small molecules (such as lactate) we have preferred to use the more general term 'metabolic repression'.

The further problem that arises is whether a single metabolic repressor controls the formation of all enzymes that are subject to the glucose effect or whether there is a specific metabolic repressor for each. The latter interpretation would be consistent with the finding of Neidhardt & Magasanik (1957) that in *Aerobacter aerogenes* the glucose effect on histidase could be relieved under conditions where myoinositol dehydrogenase remained repressed.

We have examined β -galactosidase, D-serine deaminase and L-tryptophanase in *E. coli*. These are all inducible and subject to the glucose effect. The results show that metabolic repression is indeed specific and that the repressor in each case is one of the end products of enzyme action or some substance closely related to it. The more general aspects of these findings have been discussed by McFall & Mandelstam (1963).

METHODS

Organisms. The following strains of *E. coli* K12 were used: 58-161, which had a requirement for methionine. The bacteria were inoculated into a minimal medium (see below) without methionine, and a back mutant was selected that had lost the requirement. This revertant, 58-161 (met⁺), was used in all subsequent work. The following galactose-negative strains (also K12) were kindly supplied by Dr J. Lederberg: W 3092 (lacking ATP-D-galactose 1-phosphotransferase, EC 2.7.1.6); W 3094 (lacking UDP-glucose- α -D-galactose 1-phosphate uridylyltransferase, EC 2.7.7.12); W 3805 and 4221 (lacking UDP-glucose 4-epimerase, EC 5.1.3.2). These strains will be referred to as kinaseless, transferaseless and epimeraseless respectively. The wild-type was W 3110; *E. coli*

ML 328C (lac^+ ; see Mandelstam, 1958) was also used. The organisms were grown with shaking at 35° in a synthetic medium (pH 7.2) containing phosphate and inorganic salts (Mandelstam, 1962) with glycerol or other carbon source, at a final concentration of 10 mg./ml. unless stated otherwise. The medium also contained pyridoxine hydrochloride (10 $\mu\text{g.}/\text{ml.}$). When a magnesium-limiting medium was required for experiments in a continuous-flow apparatus (Mandelstam, 1962) the concentration of MgSO_4 was reduced from 20 to 3 mg./l.

Induction of enzyme. Induction of β -galactosidase and tryptophanase in flask-culture experiments was carried out as follows. Cells in early exponential phase of growth (0.05–0.1 mg. dry wt./ml.), in glycerol or lactate medium, were treated with methylthio- β -D-galactopyranoside (500 $\mu\text{g.}/\text{ml.}$) or with L-tryptophan (300 $\mu\text{g.}/\text{ml.}$); after 1 hr. a further addition of tryptophan (300 $\mu\text{g.}/\text{ml.}$) was made to ensure continued induction. Samples (5 ml.) were taken at 30 min. intervals, usually for 2 hr. β -Galactosidase was assayed after treating the cells with toluene (Rickenberg & Lester, 1955). A unit of enzyme hydrolyses 1 μmole of o-nitrophenyl- β -D-galactopyranoside/hr.

Various attempts were made to assay tryptophanase in cell-free preparations, but all methods of disruption produced loss of activity (50–90%). The methods used included treatment with toluene, benzene or chloroform, ultrasonic disruption, and osmotic disruption of spheroplasts produced by the action of lysozyme. In no case was the lost activity restored by pyridoxal phosphate (50 $\mu\text{g.}/\text{ml.}$). The enzyme was accordingly assayed in whole cells as follows. The samples were centrifuged, washed once with 5 ml. of sodium phosphate buffer (0.05M; pH 7.0). The cells were resuspended in 5 ml. of the same buffer and a sample (1.0 ml.) was incubated at 35° with 1.0 ml. of L-tryptophan (2 mg./ml.) for a suitable period (usually 20 min.). The reaction was stopped by the addition of 3.0 ml. of modified Ehrlich's reagent (Pardee & Prestidge, 1961), and the indole colour was read at 568 $\text{m}\mu$ after being allowed to develop for 20 min. at room temperature. One unit produces 1 μmole of indole/hr.

Induction of D-serine deaminase was not carried out in flask cultures because D-serine produced a transient toxic effect (see Maas & Davis, 1950), which made the kinetics difficult to interpret. Experiments with this enzyme were therefore done in the continuous-flow apparatus (Mandelstam, 1962) with cells preinduced by growth in the presence of D-serine (100 $\mu\text{g.}/\text{ml.}$) (see below). The same concentration of D-serine was used for the continuous culture.

The enzyme was assayed in washed toluene-treated cells by the method of Pardee & Prestidge (1961). One unit produces 1 μmole of pyruvate/hr. from D-serine.

RESULTS

Dissociation of repression of β -galactosidase from that of tryptophanase and D-serine deaminase. If a common metabolic repressor existed for the three enzymes, the imposition of repressive conditions upon a preinduced culture should produce qualitatively similar results, i.e. the enzymes should be repressed more or less in parallel. Even if the three biosynthetic mechanisms differed greatly in their

sensitivity to the common repressor, in general a situation should not arise in which one enzyme is being repressed while another is being de-repressed. If such a dissociation were found, it would imply the presence of more than one repressor.

A number of experiments were carried out as follows. *E. coli* (ML 328C, lac^+) was grown in flask culture in glycerol medium containing inducers for the three enzymes (see the Methods section). The cells were centrifuged, washed and suspended in a similar medium but lacking Mg^{2+} ions. The suspension was transferred to the continuous-flow apparatus and supplied with magnesium-limiting medium (see the Methods section) so as to give a doubling time of 140 min., i.e. about one-third of the growth rate in the flask culture. Previous experiments with β -galactosidase (Mandelstam, 1962) had shown that such a limitation was sufficient to produce a significant degree of repression. The activities of the enzymes were then followed during transition to the steady state.

The specific activity of the β -galactosidase fell steadily from the beginning of the experiment (Fig. 1), but the activities of the tryptophanase and serine deaminase increased sharply at first and then fell more or less in parallel. During the first hour there was thus a clear dissociation between the repression of β -galactosidase and that of the other two enzymes.

In *E. coli* K12 it was also possible to get dissociation of the repression of β -galactosidase from that of tryptophanase and serine deaminase, but in this strain it occurred in conditions of nitrogen limitation.

Comparative repressibility of β -galactosidase and tryptophanase in the presence of various carbon sources. The apparently co-ordinated repression of tryptophanase and serine deaminase suggested that the two enzymes shared a common repressor. Now pyruvate is an end product of enzyme action in both, and it had already been reported by Freundlich & Lichstein (1960) that pyruvate inhibited the synthesis of tryptophanase. In addition, Cohn & Monod (1953) had found that galactose repressed β -galactosidase in *E. coli*. We therefore examined the possibility that the 'real' repressor in each case is one of the end products of the enzyme action, and we began with a comparison of tryptophanase and β -galactosidase in *E. coli* K12.

Bacteria growing in glycerol medium were induced with methylthio- β -D-galactopyranoside or with L-tryptophan and in many experiments with both inducers together. After 30 min. the substance being tested as a repressor was added (at a final concentration 5 mg./ml.) to a portion of the culture. Further samples were taken for analysis at 30 min. intervals.

β -Galactosidase formation was repressed by both

end products, i.e. by glucose and by galactose (Fig. 2). From the differential rate of enzyme synthesis, which is proportional to the slope of the line, it was apparent that galactose was about twice as effective a repressor as glucose. By contrast pyruvate, after the usual transitory effect obtained upon addition of any carbon source, allowed a rate of synthesis equal to that of the control culture.

For tryptophanase the pattern of repression was

Fig. 1. Dissociation of repression of β -galactosidase from that of D-serine deaminase and L-tryptophanase during magnesium deprivation. Cells (ML 328 C, lac⁺) were first grown in flask culture in glycerol-minimal medium containing inducers for the three enzymes. The bacteria were washed, suspended in a similar medium, but lacking magnesium, and transferred to a continuous-culture apparatus. Magnesium-limiting medium (containing the inducers) was supplied at a rate sufficient to give a doubling time of 140 min. to provide conditions of metabolic repression. During the first half-generation, β -galactosidase was repressed and the other two enzymes increased in specific activity. β -Galactosidase, O; D-serine deaminase, ●; tryptophanase, Δ . Initial values for the three enzymes (units/mg. dry wt) were 425, 2.75 and 1560 respectively. Culture density was 0.58 mg. dry wt./ml.

quite different, pyruvate being the most effective substrate, whereas galactose, apart from a transitory effect, had no significant repressor action; glucose had intermediate activity (Fig. 3). A number of metabolites that are utilized via the citric acid cycle were compared with pyruvate for their effect on production of tryptophanase. Pyruvate was the only compound giving more or less complete repression (Fig. 4); lactate was fairly active and succinate and malate were much less so. The same substrates when tested as repressors of β -galactosidase were found to be as ineffective as pyruvate.

Indole as another end product of tryptophanase action was also tested (at 100 μ g./ml.). It was without effect on tryptophanase production.

Comparison of repression of D-serine deaminase with that of tryptophanase. For the reasons already given, experiments with D-serine deaminase were done in the continuous-flow apparatus. The repressibility of this enzyme by pyruvate was compared with that of tryptophanase as follows. Bacteria (58-161, met⁺) were grown in glycerol medium con-

Fig. 2. Effect of galactose, glucose and pyruvate on induced formation of β -galactosidase in strain 58-161 (met⁺). Bacteria growing in glycerol-minimal medium were induced with methylthio- β -D-galactopyranoside. At the point indicated by the arrow, the culture was split into four and these additions were made: none, O; galactose, ●; glucose, Δ ; pyruvate, ■.

taining D-serine and L-tryptophan with Mg^{2+} ions as the growth-limiting factor. The flow rate was set to give a doubling time of 240 min., so that both enzymes were partially repressed. The culture was grown for 36 hr. to come to a steady state. Then the flow rate was increased to give a doubling time of 120 min. Previous experiments had shown that this would partly relieve the repression. However, at the same time pyruvic acid (neutralized with sodium hydroxide) was added directly to the culture vessel to a final concentration of 5 mg./ml. The activities of the two enzymes were then measured as the pyruvate was diluted out. Both enzymes were partially repressed, falling by about one-third in one generation (Fig. 5). The serine deaminase then recovered sharply. The tryptophanase, however, continued to fall slowly for another generation, and then it, too, recovered abruptly. This result suggested that tryptophanase was about twice as sensitive to repression by pyruvate as serine deaminase. The difference was found consistently and a similar result was obtained with glucose as the repressor; i.e. tryptophanase was the more pro-

foundly affected enzyme and it escaped from the repression about a generation later.

Galactose, succinate and lactate, which were poor repressors of tryptophanase (see above), also failed to repress D-serine deaminase significantly.

Selection and properties of a mutant with tryptophanase and β -galactosidase synthesis resistant to glucose repression. The fact that glucose and pyruvate inhibited tryptophanase production made it possible to use repression as a basis for attempting to select irrepressible mutants.

Strain 58-161 (met^+) was grown in glycerol with tryptophan as the only source of nitrogen. In this medium growth thus depended upon the ammonia liberated by tryptophanase. Addition of either glucose or pyruvate to the culture allowed the cells to go on growing for a while, using the tryptophanase which was already present. Gradually, however, the repressor produced an increasingly inhibitory effect until, at the end of about 6 hr., growth had ceased altogether. The result of an experiment with pyruvate is shown in Fig. 6; similar growth curves were obtained with glucose.

Fig. 3. Effect of galactose, glucose and pyruvate on induced formation of L-tryptophanase. Strain 58-161 (met^+) was induced with L-tryptophan (see the Methods section). Otherwise procedure and symbols are the same as those in the legend of Fig. 2.

Fig. 4. Repression of tryptophanase by pyruvate and metabolically related substances. Strain 58-161 (met^+) growing in glycerol-minimal medium was induced with tryptophan, and test substances were added to portions of the culture: none, ○; lactate, ●; malate, △; pyruvate, ▲; succinate, □.

After the cultures had been shaken at 35° for several days, mutants appeared that were able to grow in the presence of the inhibitor. Several mutants of each type were examined. Those selected from the pyruvate medium were found either to have lost the ability to use glycerol at all or else to have a greatly enhanced capacity for utilizing pyruvate, as shown by a high growth rate in a medium with pyruvate as the only source of carbon. It seems reasonable to suppose that, in these mutants, the intracellular amount of pyruvate was insufficient to repress the tryptophanase effectively. The growth of these organisms, in a medium with tryptophan as nitrogen source, was not stopped by glucose. Apparently then loss of the property of repressibility by pyruvate brings concomitant relief from the glucose effect.

In contrast with this, the mutants selected in the glucose-tryptophan medium were unable to grow in pyruvate medium until sufficient time had elapsed for selection of further resistance. In addition, when these mutants were grown in glucose-ammonium salts medium the doubling time was about 80 min., compared with 55 min. in the parent strain. These strains thus grew as badly in glucose as in succinate, lactate or other such poor carbon sources, but they grew as well on glycerol as did the parent strain. The results are consistent with the assumption that, in these mutants, there is a partial

defect early in glucose metabolism, for instance in hexokinase or one of the immediately succeeding enzymes.

The fact that in these strains pyruvate was still growth-inhibitory in a tryptophan medium whereas glucose was not obviously implied that the tryptophanase was still repressible by pyruvate but had become resistant to glucose.

When the enzymes of one of these strains were examined in detail it was found that the tryptophanase had, in fact, completely escaped from glucose repression. Furthermore β -galactosidase was also no longer repressed by glucose (Fig. 7). However, each enzyme was still sensitive to specific repression by its end product, i.e. the tryptophanase was still as repressed by pyruvate, and the β -galactosidase by galactose, as in the parent strain. Serine deaminase, when studied in the continuous-flow apparatus, had also lost sensitivity to glucose but remained repressible by pyruvate.

The enzymic behaviour of this mutant thus confirmed the conclusion that a metabolic defect had

Fig. 5. Comparison of repressibility of L-tryptophanase and D-serine deaminase by pyruvate. Strain 58-161 (met⁺) was grown in a continuous-culture apparatus in glycerol-minimal medium with L-tryptophan and D-serine present, and with magnesium as the growth-limiting factor. A single addition of pyruvate (5 mg./ml. final concentration) was made directly to the growth flask, and the specific activities of the enzymes were followed subsequently (for further details see text). Serine deaminase, O; tryptophanase, ●. Culture density was 0.56 mg./ml.

Fig. 6. Effect of pyruvate on growth of cells in a medium with tryptophan as sole nitrogen source. Strain 58-161 (met⁺) was grown in glycerol-minimal medium with the ammonium salts replaced by L-tryptophan (500 μ g./ml.). When growth had been established, the culture was divided: no addition, ●; pyruvate added (5 mg./ml.), O.

Fig. 7. Effect of glucose on enzyme induction in a strain selected for irrepressibility of tryptophanase by glucose. The mutant of 58-161 (met^+) (for details of selection see text) was grown in glycerol-minimal medium and induced with (a) tryptophan or (b) methylthiogalactoside. No addition, $\circ$; glucose added (at arrows), $\bullet$, $\blacktriangle$.

resulted in a slower utilization of glucose, so that specific repressors such as pyruvate and galactose did not accumulate sufficiently to cause repression.

This mutant thus resembles that isolated in *Aerobacter*, in which histidase is not repressed by glucose (Neidhardt, 1960).

Repression of β -galactosidase in galactose-negative mutants. In an attempt to identify the metabolic repressor of β -galactosidase more definitely, we compared repression in various galactose-negative mutants with that in the wild-type parent. Three types of mutants were examined: (1) lacking galactokinase (unable to form galactose phosphate); (2) transferaseless (unable to convert galactose 1-phosphate into UDP-galactose); (3) epimeraseless (unable to convert UDP-galactose into UDP-glucose). (A fourth type of mutation affecting galactose metabolism is the inability to form UDP-glucose; Fukasawa, Jokura & Kurahashi, 1962. This type of mutant was not examined.)

The β -galactosidase of these K_{12} strains, and that of the parent (W3110), differed from 58-161 (met^+) in being considerably repressed, even in the glycerol medium. This reduced the magnitude of the repression by glucose or galactose so that these com-

Fig. 8. Repression of β -galactosidase in galactose-negative mutants. Mutant strains lacking (a) kinase, (b) transferase or (c) epimerase (see text) were grown in lactate-minimal medium and induced with methylthiogalactoside. Further additions were made (at the arrows): none, $\circ$; galactose, $\bullet$; glucose, $\blacktriangle$.

pounds inhibited β -galactosidase synthesis by 40–50% instead of 80–90%. Lactate was therefore used as the carbon source for growth, since it is a poorer substrate and a poorer repressor and the effects of glucose and galactose are correspondingly more distinct. Otherwise the experiments were carried out as before.

With the wild-type parent and with the kinaseless and transferaseless mutants the induction curves were similar to those found previously with *E. coli* 58-161 (met^+), i.e. marked repression by both glucose and galactose, with the latter rather more active (Fig. 8). In the epimeraseless mutant galactose was still a repressor, but this strain differed markedly from the other two and the wild-type in its response to glucose, which had relatively little effect. Nevertheless, the inhibition was still significant, and when the experiment was repeated with another epimeraseless strain (W4221) the result was very similar.

DISCUSSION

Before assessing the results it is necessary to consider the possibility that the metabolic repressors exert their effect primarily by preventing uptake of the inducer and not by inhibiting the enzyme-forming systems. This problem has been considered by Freundlich & Lichstein (1960) in their study of the tryptophanase of *E. coli*. They showed that, under conditions in which glucose completely suppresses the induced formation of tryptophanase, tryptophan nevertheless prevents the formation of tryptophan synthetase and is therefore entering the cell. We have observed that the rate of degradation of tryptophan by preinduced cells is not decreased

in the presence of either glucose or pyruvate. In the experiments on β -galactosidase this factor was also taken into account, and the inducer was used at a concentration of 500 $\mu\text{g./ml.}$ (see the Methods section). This is about ten times the usual inducing concentration, and is sufficient to ensure that, even in permeaseless strains of *E. coli*, sufficient inducer enters the cells by diffusion to give a maximal rate of induction from the start. Apart from this, Mandelstam (1962) showed that metabolic repression is found equally in constitutive strains of *E. coli*, where the problem of inducer penetration does not arise.

The work was undertaken to determine whether the synthesis of inducible degradative enzymes is controlled by a common metabolic repressor or whether the regulation mechanisms are specific. The dissociation of the repression of β -galactosidase from that of the other two enzymes (Fig. 1) and the contrasting patterns of repression illustrated in Figs. 2 and 3 alike suggest that metabolic repression is specific. These Figures show that the end product of the action of an enzyme is even more effective in repressing that enzyme than is glucose itself, whereas it has no repressor effect upon an unrelated enzyme.

In a biochemically 'versatile' organism it is difficult to demonstrate formally that the end product of enzyme action is the 'real' repressor, especially when the substance concerned is so important metabolically as pyruvate. In the β -galactosidase system, the problem is more tractable because galactose is not an essential metabolite and because mutants have been isolated which lack one of the enzymes in the following sequence:

The fact that galactose represses β -galactosidase in all three types of mutant means that galactose or galactose 1-phosphate or UDP-galactose, or all three, could be active repressors. Alternatively, it might be that one of them, say galactose itself, is the active compound. Galactose 1-phosphate might then give rise to free galactose by the reverse action of kinase. This could happen even in the kinaseless strain if the block were not absolute or, more probably, by the action of one of the phosphatases that the cells are known to possess. Similarly it could be argued that either galactose 1-phosphate or UDP-galactose is the repressor.

The results of the experiment with glucose are more definitely interpretable. It is apparent that glucose is an effective repressor in the kinaseless and transferaseless strains but that loss of the epimerase leads to almost complete loss of the

glucose effect. As far as is known, the epimerase, working in reverse, is the only enzyme available in coliform bacteria for the production of galactose from glucose, and cells lacking this enzyme build a defective cell wall that contains no galactose (Nikaido, 1961). It thus appears that loss of the ability to make galactose confers almost complete insensitivity to glucose repression. This finding strongly suggests that glucose exerts its effect upon β -galactosidase synthesis via galactose. The relatively small residual repression produced by glucose in the epimeraseless strain could have at least two explanations. Either the glucose molecule resembles galactose sufficiently to function as an analogue with weak repressor activity, or the block at the epimerase level is incomplete and enough UDP-galactose is formed to give a low degree of repression. It seems clear that galactose itself or a substance closely related to it is the active metabolic repressor in the β -galactosidase system and that the glucose effect is largely abolished in cells that cannot synthesize galactose.

In the serine-deaminase and tryptophanase systems an analogous investigation would require a mutant that lacked the ability to produce pyruvate, could not utilize pyruvate and could grow without it. In the absence of such a mutant only circumstantial evidence can be adduced. This evidence consists of the finding that pyruvate is a better repressor than glucose itself, and a great deal more active than a number of metabolically related compounds (Fig. 4). Also, the mutant that utilizes glucose badly lacks the glucose repression but still exhibits repression of both tryptophanase and serine deaminase by pyruvate. The facts are consistent with, but of course do not prove, the suggestion that pyruvate or a closely related metabolite is the repressor, and that glucose acts through this substance and not as such.

If it is assumed that the three systems we have examined are typical examples of inducible degradative enzymes supplying carbon and energy we are led to the general conclusion that the repressor is either the end product of the enzyme action or is closely related to it metabolically. In this sense the repression of degradative enzymes is analogous to the end-product repression mechanism that controls the formation of many biosynthetic enzymes (see also McFall & Mandelstam, 1963).

SUMMARY

1. Metabolic repression of three inducible enzymes, β -galactosidase, tryptophanase and D-serine deaminase, has been examined in *Escherichia coli*. The repression of the first enzyme can be dissociated from that of the other two with a continuous-culture apparatus.

2. There are specific repressor effects: galactose is an effective repressor for β -galactosidase but not for the other enzymes; conversely, pyruvate represses tryptophanase and serine deaminase but not β -galactosidase.

3. In wild-type *E. coli* glucose represses all three enzymes. A mutant has been selected in which there is no glucose effect for any of the enzymes but in which each enzyme remains specifically repressible.

4. β -Galactosidase synthesis in galactose-negative mutants has been examined. In kinaseless and transferaseless strains the enzyme is repressed both by glucose and galactose. In epimeraseless strains the glucose effect is largely abolished. This indicates that the ability to form uridine diphosphate galactose is an essential part of the glucose effect.

5. The conclusion is drawn that metabolic repression of an enzyme is specifically produced by an end product of the enzyme action or some closely related compound, and that glucose acts as a repressor only when it can give rise to sufficient amounts of the specific repressor.

6. The regulation of synthesis of induced degradative enzymes by end-product repression is analogous to that found in biosynthetic pathways.

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Renal Gluconeogenesis

2. THE GLUCONEOGENIC CAPACITY OF THE KIDNEY CORTEX OF VARIOUS SPECIES*

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The occurrence of renal gluconeogenesis is well established from experiments on tissue slices and the perfused organ (for references see Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963), but no systematic measurements have so far been reported on the gluconeogenic capacity of the renal cortex of different species. The present paper reports such measurements obtained with the procedure described by Krebs *et al.* (1963). An excess of various gluconeogenic substrates was added to

kidney-cortex slices so that lack of substrate was not a limiting factor.

EXPERIMENTAL

Slices of kidney cortex, suspended in the medium of Krebs & Henseleit (1932), were used throughout. The details of the procedure were essentially as described by Krebs *et al.* (1963). Glucose was determined by the glucose-oxidase method. Glycogen was not determined in every experiment because its contribution to gluconeogenesis proved to be very slight in a few test cases. Incubation was for 1 hr. at 40°. The concentration of added substrates was 10 mM. When three or more individual kidneys of the same

* Part 1: Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963).