dent. Keto groups are clearly essential in the binding of carotenoid to apocrustacyanin.

Kuhn & Lederer (1933) and Ball (1944) suggest that the blue and green colours of astaxanthin proteins result from enolization of the carotenoid and resultant salt formation between basic protein groups and the tetravalent carotenoid anion. Platt (1959) has stressed that polarization of carotenoids can cause large bathochromic shifts in their absorption spectra. The formation of purple chromoproteins from apocrustacyanin and astacene, which cannot enolize in the 4- and 4′-positions, and from canthaxanthin, which has no 3- or 3′-substituents, suggests that the observed bathochromic shifts result simply from polarization due to interaction with a specific site on the protein. Isolation of astacene from natural sources has been attributed to oxidation of astaxanthin during isolation procedures (cf. Goodwin, 1954). On the basis of the present study, a more careful examination of these cases may be warranted.

Naturally occurring canthaxanthin–proteins (Lee, 1966*a*,*b*) have absorption maxima further to the red than has α-crustacyanin; it is not known how far the quaternary structure of these depends on the structure of the prosthetic group.

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Mechanism for the Stimulation *in vivo* of Hepatic Gluconeogenesis by Glucagon

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Glucagon has a well-established glycogenolytic action on liver. This effect is mediated by 3′,5′-(cyclic)-AMP, which is formed by the interaction of glucagon with the membrane-bound adenylate-cyclase system (Sutherland, Øye & Butcher, 1965). Glucagon not only stimulates glucose formation from glycogen, but also increases the rate of glucose synthesis from C_3 precursors (Struck, Ashmore & Wieland, 1965; Garcia, Williamson & Cahill, 1966; Exton & Park, 1966). However, the mechanism for the gluconeogenic effect of glucagon has not yet been clearly defined.

In addition to activating the phosphorylase system, glucagon and 3′,5′-(cyclic)-AMP have been shown to activate a triglyceride lipase of adipose tissue (Rizack, 1964) and of liver (Bewsher & Ashmore, 1966). Since fatty acids can stimulate gluconeogenesis when added *in vitro* to the perfused rat liver (Struck *et al.* 1965; Williamson, Kreisberg & Felts, 1966*b*) or to kidney-cortex slices (Krebs, Speake & Hems, 1965), the possibility arises that the gluconeogenic effect of glucagon is mediated by an enhanced availability of fatty acids.

To test this hypothesis, and some of the theories on the control of gluconeogenesis that have been advanced recently (Krebs, 1964; Walter, Paetkau & Lardy, 1966; Newsholme & Underwood, 1966), glucagon was administered to fed rats *in vivo*. The

liver was subsequently analysed for most of the metabolic intermediates and cofactors known to be involved in the gluconeogenic pathway.

Methods. Male fed rats of the Holtzman strain (210–230g.) were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50mg./kg. body wt.) and the femoral vein was cannulated. Crystalline insulin-free glucagon (lot no. 258-234B-167-1; courtesy of Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A.) was dissolved in Krebs bicarbonate buffer (Krebs & Henseleit, 1932) and administered intravenously at the rate of 50μg./min. for the first minute, followed by 5μg./min. for a total period of 30min. Control rats were treated similarly with infusions of Krebs bicarbonate buffer. The livers were rapidly frozen *in situ* with tongs precooled in liquid N_2. Portions of powdered tissue (800mg.) were extracted once at $-10°$ with 8% (v/v) $HClO_4$ in 40% (v/v) ethanol, and once at 0° with 6% (v/v) $HClO_4$. The combined supernatants were neutralized in the cold to pH 5·5 with 3M-K_2CO_3 containing triethanolamine (0·5M), and the precipitated $KClO_4$ was removed by centrifugation. Metabolic intermediates and cofactors were measured enzymically by the fluorimetric procedures previously described (Williamson, 1965).

Results and discussion. The occurrence of enhanced gluconeogenesis after the administration of glucagon to rats *in vivo* was tested by measuring the incorporation of [^{14}C]bicarbonate into plasma glucose (Wagle & Ashmore, 1963). Glucagon produced a five-fold increase of [^{14}C]glucose production after 30min. (J. Ashmore & J. R. Williamson, unpublished work). The effect of glucagon on the tissue content of 23 metabolic intermediates in livers from fed rats is presented in Table 1. All the changes shown, except for lactate and aspartate, were statistically significant ($P < 0·05$). The concentrations of pyruvate and oxaloacetate in the glucagon-treated group decreased relative to the controls, whereas all other intermediates in the linear gluconeogenic sequence from lactate to glucose increased.

The rapidity of the gluconeogenic effect of glucagon suggests that it is due to a specific activation of one or more 'pacemaker' or control enzymes of the gluconeogenic sequence (Krebs,

Table 1. *Effects of glucagon on the hepatic concentrations of metabolic intermediates*

Glucagon was administered to fed rats by intravenous infusion at the rate of 50μg./min. for the first minute followed by 5μg./min. for a total time of 30min. The livers were rapidly frozen *in situ* with tongs precooled in liquid N_2. Values are the mean ± S.E.M. with six rats in each group.

Intermediate	Concn. in control (mμmoles/g. dry wt.)	Percentage change with glucagon (% of mean control value)
Lactate	2352±293	91±6
Pyruvate	207±18	61±5
Oxaloacetate	14·7±1·5	73±10
Malate	1380±75	142±5
Phosphoenolpyruvate	338±22	201±14
2-Phosphoglycerate	90±5	151±10
3-Phosphoglycerate	721±51	218±12
Glyceraldehyde 3-phosphate	9·0±0·8	144±12
Dihydroxyacetone phosphate	57·5±4·1	130±6
α-Glycerophosphate	703±49	205±21
Fructose 1,6-diphosphate	11·9±1·4	179±18
Fructose 6-phosphate	243±21	214±13
Glucose 6-phosphate	1140±115	217±13
Glucose 1-phosphate	28±1	406±39
Glucose	22,520±570	152±14
NADH	377±29	120±2
ATP	10,480±200	84±3
ADP	2,460±256	186±11
AMP	406±55	215±21
Citrate	886±88	131±16
Acetyl-CoA	165±11	160±13
Glutamate	12,240±860	44±3
Aspartate	2,770±308	93±9
Lactate/pyruvate ratio	11·9±1·4	144±9
Malate/oxaloacetate ratio	90±7	195±22
α-Glycerophosphate/dihydroxyacetone phosphate ratio	12·3±0·6	156±14
ATP/ADP ratio	4·83±0·41	41±4

1964). One such site, consistent with the observed changes of the metabolic intermediates, involves an activation of pyruvate carboxylase (see Utter & Keech, 1963) by the elevated tissue concentrations of acetyl-CoA (Table 1; Williamson, Herczeg, Coles & Danish, 1966*a*). The failure of oxaloacetate concentrations to rise when pyruvate-carboxylase activity is enhanced may be explained by the conversion of part of the oxaloacetate formed from pyruvate during gluconeogenesis into malate (eqns. 1–3).

$$\text{Pyruvate} + \text{ATP} + \text{CO}_2 \xrightarrow[\text{Acetyl-CoA}]{\text{Mg}^{2+}} \text{oxaloacetate} + \text{ADP} + \text{P}_i \quad (1)$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{malate} + \text{NAD}^+ \quad (2)$$

$$\text{Sum: Pyruvate} + \text{ATP} + \text{CO}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{malate} + \text{NAD}^+ + \text{ADP} + \text{P}_i \quad (3)$$

The equilibrium of eqn. (2) is pushed from left to right because of a shift of the redox potential of the NAD system to a more negative value (see Klingenberg. & Bücher, 1960; Henning, Stumpf, Ohly & Seubert, 1966). This is evidenced by a rise of total NADH concentration in the liver after glucagon administration and by an increase in the ratios of the redox substrate couples (Table 1). Thus the metabolic product of the pyruvate-carboxylase reaction may be regarded as the sum of malate and oxaloacetate. The rise in phosphoenolpyruvate concentration in association with a fall in oxaloacetate concentration may be indicative of an activation of phosphoenolpyruvate carboxykinase. Alternatively, these substrate changes may reflect an inhibition of pyruvate kinase by an allosteric modifier, such as NADH, as suggested by Weber, Singhal, Stamm & Srivastava (1965).

The probable sequence of events after glucagon administration may be summarized as follows: 3′,5′-(cyclic)-AMP concentrations in the liver rise (R. W. Butcher, unpublished work, cited by Exton & Park, 1966), resulting in the activation of phosphorylase and triglyceride lipase. The tissue concentrations of the hexose monophosphates increase as the result of increased glycogenolysis, and glucose production increases owing to the activity of glucose 6-phosphatase. Fatty acid availability is enhanced by the increased mobilization of triglyceride in both the liver and adipose tissue. Increased oxidation of fatty acids causes an increased rate of ketone-body formation and an elevation of the tissue concentrations of acetyl-CoA and fatty acyl-CoA (Williamson *et al.* 1966*a*), and of reduced nicotinamide nucleotides. Pyruvate oxidation is decreased (Walter *et al.* 1966), owing either to an inhibition of pyruvate oxidase by acetyl-CoA (Garland & Randle, 1964) or to a competition for CoA between pyruvate oxidase and the fatty acid-oxidizing system. Conversion of pyruvate into oxaloacetate, on the other hand, is increased owing to the stimulation of pyruvate carboxylase by acetyl-CoA. Malate formation is promoted by the increased supply of NADH from fatty acid oxidation and malate passes to the cytoplasmic compartment, where it is partly reoxidized to oxaloacetate and subsequently converted into phosphoenolpyruvate (Shrago & Lardy, 1966). Reducing equivalents supplied to the cytoplasm in this manner, or by one of the other possible hydrogen redox couples (Borst, 1964), are used in the reductive step of gluconeogenesis during the conversion of 1,3-diphosphoglycerate into glyceraldehyde 3-phosphate. In addition, energy needed for the enhanced rate of gluconeogenesis is generated in the electron-transport chain by the oxidation of reduced flavine and nicotinamide nucleotide formed during fatty acid oxidation.

In experiments with isolated livers from unfed rats perfused with 10mM-alanine, Williamson *et al.* (1966*b*) proposed that the enhanced rate of gluconeogenesis on the addition of fatty acids was caused by facilitation of the triose phosphate-dehydrogenase step owing to the elevated cytoplasmic NADH concentrations. This conclusion was based on the observation that fatty acids caused a relative depletion of intermediates in the sequence from pyruvate to 3-phosphoglycerate and a relative accumulation of intermediates from triose phosphate to glucose. Since livers from unfed rats were used for the perfusion studies, it is possible that acetyl-CoA concentrations were sufficiently elevated so that pyruvate carboxylase was not the major control site under the conditions of the experiment. Evidently, one of a number of enzyme sites may control the rate of glucose production, depending on the nutritional state of the liver and on the availability and nature of gluconeogenic precursors. The dependence of gluconeogenesis on substrate supply is difficult to assess *in vivo*, but it appears unlikely, in view of the observed fall in the concentration of glutamate and lack of a rise in the concentrations of lactate and aspartate (Table 1), that an increased supply of precursors is responsible for the flux increase in the present experiments.

In addition to providing evidence in favour of control by acetyl-CoA at the pyruvate-carboxylase site after glucagon administration, the present results provide evidence against adenine nucleotide control of the activities of phosphofructokinase and fructose 1,6-diphosphatase as a trigger for gluconeogenesis (Gevers & Krebs, 1966; Newsholme & Underwood, 1966). As shown in Table 1, the concentrations of ADP and AMP increased after glucagon administration. These changes would tend to facilitate phosphofructokinase and inhibit fructose 1,6-diphosphatase, resulting in an inhibition of gluconeogenesis. The rise of the citrate

concentrations may inhibit phosphofructokinase, but the increase of citrate is small relative to that of ADP. It appears probable that control at these sites is rather insensitive at physiological substrate concentrations, and may serve primarily to shut off gluconeogenesis and enhance glycolysis under conditions of severe ATP deficiency, such as anoxia. The extent *in vivo* of the energetically wasteful cycle of fructose diphosphate formation by phosphofructokinase and destruction by fructose diphosphatase, and also possible uncoupling of oxidative phosphorylation by fatty acids, is not known. However, calculations by Rognstad & Katz (1966) of energy balances in adipose tissue, and the excess of oxygen consumption of kidney-cortex slices producing glucose in the presence of acetoacetate (Krebs *et al.* 1965), suggest the possibility that the energy cost of gluconeogenesis may be considerably higher than the theoretical 6 molecules of ATP/molecule of glucose. The changes of enzyme patterns observed in the livers of diabetic, starved and cortisone-treated rats (Weber *et al.* 1965) presumably serve to increase the efficiency as well as the capacity of gluconeogenesis, but they are too slow to account for its initiation.

Adrenaline probably acts on liver in a similar manner to glucagon (Exton, Jefferson, Butcher & Park, 1966; Exton & Park, 1966). The present studies suggest that the effect of these hormones on gluconeogenesis is mediated by the release of fatty acids. It appears that gluconeogenesis is 'switched on' primarily by the rise in acetyl-CoA and NADH concentrations, which facilitate, respectively, the pyruvate-carboxylase and triose phosphate-dehydrogenase steps. Whether glycolysis is effectively 'switched off' by analogous control mechanisms requires further clarification.

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Mechanism of Action of Bovine Pancreatic Ribonuclease

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The binding of nucleotides to ribonuclease may be investigated by spectrophotometric methods. The spectra of purine and pyrimidine nucleotides are all perturbed on interaction with the active site of ribonuclease, with a lowering of the extinction of the base, and, sometimes, a small bathochromic shift. These changes are attributed to the nucleotide rather than the protein because their nature varies with the character of the base. For uridine nucleotides (2′-, 3′- and 5′-) the position of maximum decrease in the