July–August without any appreciable change in oil content suggesting the possibility of preferential removal of vitamin A as compared with the oil.

5. Studies on the distribution of vitamin A and of oil showed that whereas the oil was diffused more or less evenly throughout the fish, the vitamin A reserves are concentrated particularly in the liver, with somewhat smaller proportions in the pyloric caeca and in the body (male fish only).

6. It was found that although the percentage of oil increased with increasing size of liver, the contents of vitamin A, cholesterol, total unsaponifiable matter and lipid phosphorus in the liver were relatively unaffected.

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**Efficiency of Oxidative Phosphorylation During the Oxidation of Pyruvate**

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Krebs, Ruffo, Johnson, Eggleston & Hems (1953) have recently measured the value of the ratio

\[
\text{equivalents of organic phosphate formed / atoms of oxygen consumed}
\]

('phosphorylation quotient') for the reactions

\[\text{x-ketoglutarate} \rightarrow \text{succinate and succinate} \rightarrow \text{fumarate plus malate}.\]

The rate of formation of organic phosphate was determined by measuring the rate of incorporation of inorganic \(^{32}\text{PO}_4\) into adenosine-triphosphate (ATP). The present paper is concerned with measurements, by the same procedure, of the phosphorylation quotient for the reaction

\[\text{pyruvate} \rightarrow \text{acetate and for the complete oxidation of pyruvate}.\]

**EXPERIMENTAL**

Preliminary experiments on liver and muscle suspensions prepared according to Krebs *et al.* (1953) showed that pyruvate, unlike the intermediates of the tricarboxylic acid cycle, did not maintain ATP in the suspensions. A more suitable material for the study of the oxidative phosphorylation associated with the oxidation of pyruvate was a suspension of washed subcellular particles of sheep kidney cortex, reinforced by cofactors, as previously described (Bartley, 1953). These were used in the present investigation. They were prepared from sheep kidneys within 3 hr. of collection and were used immediately. On keeping the tissue for 24 hr. at 0° in a mixture of frozen and liquid 0-9% (w/v) KCl the ability to carry out oxidative phosphorylation was reduced, although respiration remained unimpaired.

If the rate of incorporation of orthophosphate into ATP is to be maximal, ADP, as phosphate acceptor, must continuously be generated. The continuing formation of ADP from ATP can be brought about by the endogenous phosphatases, but, as shown by Potter & Recknagel (1951), the phosphatase activity of mitochondria separated in sucrase solutions is low. Active phosphatase may be liberated from the mitochondria by the action of KCl as shown by Berthet & de Duve (1951), or by the addition of nuclei as shown by Potter, Lyle & Schneider (1951). For these reasons the 'cyclophorase' type of preparation containing nuclei was
chosen. Unless otherwise stated, the 'R₃ residues' were used (Bartley, 1953). They were diluted with 0-9 % (w/v) KCl to give about 70 mg. dry wt./2·0 ml. of suspension.

This type of subcellular preparation, made in a Waring Blender, was satisfactory in promoting the turnover of ATP but is subject to a number of disadvantages; firstly the ability of the mitochondria to phosphorylate may be impaired; secondly the nuclei and some 'microsomes' that are mixed with the mitochondria can contribute to the O₂ uptake without concomitant phosphorylation. For these reasons the phosphorylation quotients obtained are probably somewhat too low.

The suspensions were incubated at 20° in a series of conical manometric Warburg vessels, usually ten, which contained the same mixture, but were incubated for different periods. Before the suspensions were prepared the following solutions were measured into the main compartments of the vessels: 0·5 ml. 0·02 m-sodium ATP, 0·2 ml. 0·1 M-phosphate buffer pH 7·4, 0·2 ml. 0·1 M-NaHCO₃, 0·2 ml. 0·02 M-MgSO₄, 0·1 ml. 0·1 M-sodium pyruvate, 0·2 ml. 1·86 x 10⁻⁴ M-cytochrome and 0·5 ml. water. The side arm contained 0·1 ml. of a solution of KH₂PO₄ in 0·9 % (w/v) KCl, the centre well 0·2 ml. 2 N-NaOH and filter paper. The vessels were kept in an ice-water mixture until the suspension was ready, and 2 ml. of the suspension (R₃ residue in 3 parts 0·9 % (w/v) KCl) were measured into each vessel; 0·5 ml. 30 % (w/v) trichloroacetic acid was added to one sample to stop enzymic activities, and the contents of the side arm were tipped in to give the zero value. The other cups were attached to the manometers, quickly gassed with O₂ and placed in a rack in such a way that the vessels were immersed in a trough of ice water. At zero time the contents of the main compartment and the side bulb of the first vessel were mixed and the manometer was shaken in the water bath at 20°. This procedure was repeated with the rest of the vessels at 30 sec. intervals. The first manometer was taken from the bath after 2 min. incubation, the vessel was removed from its manometer, placed in a bath of ice and water and 0·5 ml. 30 % (w/v) trichloroacetic acid was added. The procedure was repeated with other vessels after 3, 4, 6, 8, 10, 20, 30 and 40 min. incubation. The contents of the cups were centrifuged for 10 min. at 0 ° and the supernatant was stored at -14° until it could be analysed. The trichloroacetic acid extract was used for the quantitative determination of orthophosphate, ATP and pyruvate and for the measurement of the radioactivity of the phosphate fractions. The O₂ uptake was measured in the vessels incubated for 20, 30 and 40 min. The average rates were used in the calculation of the phosphorylation quotient.

Separation and estimation of the phosphate fractions. As the added inorganic orthophosphate and ATP constituted more than 95 % of the total phosphate of the trichloroacetic acid extract, other phosphates could be ignored. The separation of inorganic phosphate and of ATP was achieved by ascending paper chromatography using the formic acid-isopropyl ether mixture of Hanes & Isherwood (1949). The papers, Whatman no. 1, 45 x 19 cm., were washed in a solution of ethylenediaminetetraacetic acid as described by Eggleston & Hems (1952) and 0·125 ml. of the solution to be chromatographed was placed with an Agla syringe (Burroughs Wellcome Ltd.) along a continuous line 5 cm. long at the start of the chromatogram. It was dried by a current of warm air from a hairdryer. The chromatograms were developed from 2 to 4 hr. After drying the bands were located by the molybdate spray of Hanes & Isherwood (1949). In some experiments ethyl ether was used instead of isopropyl ether in the solvent mixture, because with this solvent separation of inorganic orthophosphate from adenosinepolyphosphates could be achieved in 30 min.

The blue bands corresponding to the ATP and the inorganic orthophosphate, usually about 7 x 3 cm. were cut out from the paper and put into test tubes graduated at 10 ml., together with 2·5 ml. of the ashing fluid of Hanes & Isherwood (1949). The paper strips were wet ashed, the volume was made up to the mark with water, and after mixing 2 ml. samples of the fluid were analysed for phosphate and radioactivity. The paper area between the adenosinepolyphosphates and the inorganic orthophosphate was similarly ashed for determination of the radioactivity of other organic phosphates between these areas. A measured area of about 7 x 4 cm. was cut from the front of the paper and analysed for the phosphate content of the paper.

To test for the presence of phosphate compounds other than adenosinepolyphosphates and inorganic orthophosphate 25 μl. samples of the filtrate were developed by descending paper chromatography with the p-toluene-
sulphonic-tert.-amyl alcohol-water mixture of Hanes & Isherwood (1949). After 17 hr. all the phosphate compounds tested (ATP and ADP, adenylic acid, thiaminediphosphate, thiaminemonophosphate, hexosediphosphate, pyrophosphate, orthophosphate, phosphopyruvate, phosphoglyceric acid, pyridoxalphosphate) were separated, except ATP and ADP which travelled together. The spots were located with the molybdysalt spray, cut out and analysed as described. No detectable amounts of phosphate other than (ATP and ADP) or orthophosphate were found in the suspensions of tissue particles.

The phosphate content of the wet-ashed material was estimated by the method of Berenblum & Chain (1938). The use of a series of funnels of the shape shown in Fig. 1 was found useful when large numbers of estimations were required. The isobutanol layer was run off after the second sulphuric acid washing and was stored together with the ethanol washings of the funnel until they could be analysed in the spectrophotometer. The isobutanol extracts showed no change after 3–4 days' storage. Immediately before the reading was taken the colour was developed by the addition of 0-1 ml. of the stannous chloride solution (10 g. in 25 ml. conc. HCl). The solution was made up to volume with pure absolute ethanol, mixed, and the colour was read in the Beckman spectrophotometer at 750 mμ. No redistillation of the isobutanol (commercial grade containing 85% isobutanol) and ethanol used was required as the green colour described by Eggleton & Hems (1952) did not occur.

Radioactivity measurements were carried out on 2 ml. samples of the paper digests diluted to 10 ml. A β counting tube, liquid type M6 (20th Century Electronics) was used.

Pyruvic acid. This was estimated either by the specific extraction method of Friedmann & Haugen (1943) or by the carboxylase method of Krebs & Johnson (1937), which was slightly modified because the solutions to be analysed contained trichloroacetic acid. The trichloroacetic acid extract (1 ml.) was put in one arm of a manometer vessel of the type shown in Fig. 2. To it was added 0-1 ml. of aqueous 0-02% (w/v) bromocresol green and enough NaOH to bring the pH to about 4-7. The total volume was brought to 5-0 ml. with water. In the other arm of the vessel was put 3-0 ml. yeast carboxylase, which had been brought to pH 4-7 with 3ml-acetate buffer and stored at –14° for at least 2 weeks. Yeast carboxylase at this pH may be stored at –14° for at least 9 months without loss of activity. After about the first fortnight of cold storage the blank CO₂ output of the carboxylase becomes negligible. The gas output after mixing was usually complete after about 60 min. Glucose did not interfere. Colorimetric and manometric methods agreed to within 5%.

Special chemicals. Barium ATP was prepared according to LePage (1949) and the preparation of the sodium salt and the estimation of its purity was carried out by the method of Eggleston & Hems (1952); 86% of the total P in the sample was present as ATP and 11% as ADP.

Pyruvic acid was redistilled from a commercial sample. The sodium pyruvate used as a standard in the colorimetric method was a pure sample kindly supplied by Dr S. R. Euldten.

RESULTS

Phosphorylation quotient during complete oxidation of pyruvate. As previously described (Bartley, 1953), complete oxidation of pyruvate to carbon dioxide and water takes place in suspensions of washed sheep-kidney particles when the pyruvate concentration is low (0-0025 m), sodium bicarbonate (0-075 m) is present, and an amount of the particles equivalent to 70–80 mg. dry wt. is used in 4 ml. The concentrations of ATP and inorganic phosphate and the distribution of radioactivity after various periods of incubation under these conditions are shown in Table 1. The data indicate that the initial concentration of ATP was maintained and that incorporation of radioactivity into the ATP occurred. The rate of incorporation v, calculated by the formula of Krebs et al. (1953), was reasonably constant for the initial period (8 min.) and subsequently dropped. The phosphorylation quotient for the initial period was about 2-2. In three similar experiments the initial quotient was between 2-1 and 2-6, average 2-2. The addition of 0-0005 m cocarboxylase lowered the phosphorylation quotient to 1-6.

Phosphorylation quotient for the reaction pyruvate → acetate. On reduction of the bicarbonate concentration, or increase of the pyruvate concentration above 0-0025 m, the suspension oxidizes pyruvate incompletely. The ratio O₄ absorbed/pyruvate removed (O₄/pyruvate ratio) falls and acetate appears as an end product (Bartley, 1953). A series of measurements of the rates of incorporation of radioactivity into the added ATP was carried out under conditions when the O₄/pyruvate ratio varied between 0-5 and 1-5. The phosphorylation
Table 1. Incorporation of radioactivity into ATP during the oxidation of pyruvate by a sheep-kidney suspension
(For details see text. Oxygen uptake 0.894 μg. atom/min. Ratio O₂/pyruvate was 2.5 throughout.)

<table>
<thead>
<tr>
<th>Time of incubation (min.)</th>
<th>Orthophosphate in vessel (μmoles)</th>
<th>Labile P* of ATP in vessel (μmoles)</th>
<th>Radioactivity in chromatogram fractions (% of total)</th>
<th>Specific activity of orthophosphate (μmoles/min.)</th>
<th>Rate of exchange of P†</th>
<th>Ratio: P exchanged atom O₂ uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Orthophosphate + ATP + Rest of the paper</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Orthophosphate = x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22.80</td>
<td>11.34</td>
<td>100-00</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>28.00</td>
<td>11.22</td>
<td>89-00</td>
<td>10.54</td>
<td>0</td>
<td>0.242</td>
</tr>
<tr>
<td>3</td>
<td>27.00</td>
<td>11.30</td>
<td>84-20</td>
<td>15.50</td>
<td>0</td>
<td>0.441</td>
</tr>
<tr>
<td>4</td>
<td>27.04</td>
<td>11.90</td>
<td>79-60</td>
<td>20.20</td>
<td>0</td>
<td>0.577</td>
</tr>
<tr>
<td>6</td>
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<td>75-20</td>
<td>24.27</td>
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<td>0.681</td>
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<tr>
<td>8</td>
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<td>0.787</td>
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<tr>
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<td>0.880</td>
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<tr>
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<td>67-30</td>
<td>31.90</td>
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<td>0.966</td>
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<tr>
<td>30</td>
<td>22.84</td>
<td>13.30</td>
<td>66-70</td>
<td>31.90</td>
<td>1.40</td>
<td>0.829</td>
</tr>
<tr>
<td>60</td>
<td>26.28</td>
<td>13.16</td>
<td>65-70</td>
<td>32.86</td>
<td>1.44</td>
<td>1.000</td>
</tr>
</tbody>
</table>

* Phosphates are calculated from the amounts recovered from the paper chromatogram. The quantities of ATP, orthophosphate and radioactivity added to each vessel were only approximately equal.

† Calc. from eqn. v = \( \frac{ab}{a+b} \ln \frac{b}{a} \).

Fig. 2. Plot of phosphorylation quotient against O₂/pyruvate (ratios calculated from these experiments are plotted against the corresponding O₂/pyruvate ratio. This gave a phosphorylation quotient of 4.6 (expt. 0.4), when the O₂/pyruvate ratio was 2.5 to 2.8.)
barrier would exist to the free diffusion of carbon dioxide. These results may explain the inability of oxaloacetate to replace bicarbonate completely in the oxidation of pyruvate to carbon dioxide and water.

*Phosphorylation at pH 7·8.* At pH 7·0 pyruvate in concentrations above 0·0005 M is oxidized almost quantitatively to acetate, provided that bicarbonate or intermediates of the tricarboxylic acid cycle are not added, the O₂/pyruvate ratio being 0·5. At a higher pH or lower pyruvate concentrations some complete oxidation occurs without the addition of bicarbonate or other catalysts (Bartley, 1953).

The study of oxidative phosphorylation at pH 7·8 showed the formation of a phosphate compound running on the paper chromatogram between ATP and orthophosphate when a p-toluenesulphonic acid-tert.-amyl alcohol-water mixture was used as the solvent; the specific activity was about 50% of that of ATP-phosphorus. The identity of the substance has not been definitely established but the position on the chromatogram suggests that it is pyrophosphate. Pyrophosphate was never detected in experiments at lower pH when the O₂/ pyruvate ratio was of the same order (0·87) as that obtained at pH 7·8. This is in agreement with the findings of Lehninger & Smith (1949) and Cross, Taggert, Covo & Green (1949) that pyrophosphate did not accumulate at pH 7·4 in the absence of fluoride. The accumulation of a third phosphate made the formula of Krebs et al. (1953) inapplicable.

*Phosphorylation in unfractionated suspensions of subcellular particles.* To investigate whether the fractionation of the suspensions by centrifugations and washing damages the mechanism of phosphorylation, experiments were carried out with unfractionated suspensions of subcellular particles made from 50 g. of kidney cortex with 200 ml. of 0·9% (w/v) potassium chloride. 2·0 ml. of this preparation were diluted with 2 ml. substrate solution. Rapid incorporation of radioactivity into the ATP fraction was obtained. No breakdown of ATP occurred and the oxygen uptake was linear with time; the calculated average phosphorylation quotient was 1·3.

The oxygen uptake was much greater than that required for the complete oxidation of the pyruvate removed, the O₂/pyruvate ratio being approximately 5. Thus substrates other than pyruvate were oxidized.

When the suspension was diluted with an equal volume of 0·9% (w/v) potassium chloride the oxygen uptake was less than required for the complete oxidation of the pyruvate removed, the O₂/pyruvate ratio being 2·0.

Incubation of an unfractionated suspension without added substrate but with the other standard additions showed that ATP was not maintained under these conditions. During the first 10-15 min. of incubation the ATP concentration fell about 20%. An approximate estimate of the phosphorylation quotient, ignoring the fall of the ATP concentration, gave a value of 0·82 (range 0·63-1·04).

As the oxygen uptake during the oxidation of pyruvate by unfractionated suspensions was greater than was required for the complete oxidation of the pyruvate removed, the distribution of oxidative capacity between the particulate and liquid fractions of a kidney suspension was measured. The suspension was centrifuged at 2000 g for 15 min. to sediment the particles, and the supernatant was poured off. The measurement of oxygen consumption indicated that 54% of the total oxygen uptake was associated with the particulate fraction. As the ability to carry out oxidative phosphorylation is associated with the particles in the suspension (Green, Atchley, Nordman & Tepley, 1949; Still & Kaplan, 1950; Harman, 1950; Green, 1951; Harman & Feigelson, 1952), it is only their oxygen consumption that is relevant to the phosphorylation quotient. Recalculation of the phosphorylation quotient for the whole suspensions on this basis gave a value of 2·42 for the complete oxidation of pyruvate and 1·52 for the endogenous substrate. The value of 2·42 is in good agreement with the results obtained with the washed suspensions. The value of 1·52 obtained when no substrate was added to the suspension would indicate that endogenous respiration does not originate from the oxidation of pyruvate alone.

**DISCUSSION**

A phosphorylation quotient of 4 for the oxidation of pyruvate to acetate is in agreement with the value postulated for the analogous oxidative decarboxylation of α-ketoglutarate (Krebs et al. 1953; Hunter & Hixon, 1949).

Previous values for the phosphorylation quotient with pyruvate as substrate vary between 2·5 (Cross et al. 1949) and about 3 (Lardy & Wellman, 1952; Ochoa, 1943). Cross et al. and Lardy did not measure the amount of pyruvate oxidized, and it is uncertain whether pyruvate was completely oxidized. In Ochoa’s experiment the O₂/pyruvate ratio was 2·5, indicating complete oxidation. The phosphorylation quotient measured was 1·9, and a value of 3·1 was arrived at by making corrections of questionable validity for dephosphorylation.

A phosphorylation quotient of 3 is expected for the complete oxidation of pyruvate (see Ochoa & Stern, 1952). The reason for the lower values of 1·9, 2·1-2·6 obtained in the present experiments cannot be satisfactorily explained. It is likely that the observed value does not represent the efficiency in the intact tissues, and that some efficiency is lost by the treatment of the material.
SUMMARY

1. The phosphorylation quotient
   (equivalents of organic phosphate formed
   atoms of oxygen consumed)
   associated with the oxidation of pyruvate by
   suspensions of washed subcellular particles
   of kidney cortex was calculated from measurements
   of the rate of incorporation of $^{32}$P into adenosinetri-
   phosphate.

2. When pyruvate, in the presence of bicarbonate,
   was completely oxidized (as indicated by an
   $O_2$/pyruvate ratio of 2-5) the average phosphorylation
   quotient was 2-23 (range 2-08-2-6).

3. When pyruvate was incompletely oxidized
   phosphorylation quotients up to 3-92 were obtained.
   From the regression line obtained by plotting
   phosphorylation quotients against the $O_2$/pyruvate
   ratio, a phosphorylation quotient of 4 was obtained
   for the oxidation of pyruvate to acetate.

4. Either bicarbonate, oxaloacetate or a pre-
   curser of oxaloacetate must be present if pyruvate is
   to be oxidized beyond acetate. The average phospho-
   rylation quotient in the presence of fumarate
   (1-7) or oxaloacetate (2-13) was lower than that
   obtained with bicarbonate. Unfractionated sus-
   pendions completely oxidizing pyruvate gave a
   phosphorylation quotient of 1-3. Reasons for
   phosphorylation quotients below the expected
   value of 3 are considered.

5. In presence of 0-001 M-fumarate or oxalo-
   acetate, pyruvate (0-0025 M) was oxidized beyond
   the stage of acetate ($O_2$/pyruvate ratio greater than
   0-5) but was not completely oxidized.

6. At pH 7-8, a phosphorus-containing com-
   pound other than adenosinetriphosphate or ortho-
   phosphate, probably pyrophosphate, was formed.

I wish to thank Prof. H. A. Krebs, F.R.S., for his help and
advice and Miss B. Dickinson for technical assistance.

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Changes in Lens During the Formation of X-ray Cataract in Rabbits

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Experimental work on cataract is directed towards
finding some change which precedes or occurs early
in opacity formation. Work has been hampered by
lack of suitable experimental material, and though
there is considerable information about the end
results of senile cataract (Bellows, 1944), little is
known of the changes occurring in animal lenses
during the production of experimental cataract.

The chemistry of the two lenses of a single normal
animal seems to be very similar (Bellows, 1944), just
as their weights are practically identical. It is
therefore an advantage to be able to produce
cataract in one lens only, leaving the second as a
control. We have chosen to irradiate the right eye
of rabbits and thus produce a cataract in this
eye while barely affecting the other. Irradiation