# The Biochemical Basis of Phagocytosis. IV. Some Aspects of Carbohydrate Metabolism During Phagocytosis*

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The effect of the phagocytic act on the metabolism of glucose has been studied in detail in polymorphonuclear leukocytes from guinea pigs. The concentration of glucose did not affect the pattern of $C^{14}O_2$ production from glucose-1-$C^{14}$ or glucose-6-$C^{14}$ within the limits studied (1.7–17 mM). The time after commencement of phagocytosis at which measurements were made with respect to oxygen consumption, glucose utilization, lactate production, or the pattern of $C^{14}O_2$ production from specifically labeled glucose substrates was found to be a critical factor. Artificial electron acceptors such as methylene blue or phenazine methosulfate caused massive increases in oxygen uptake of resting cells and in the conversion of glucose C-1 to $CO_2$ compared to the conversion of glucose C-6 to $CO_2$. The addition of pyruvate to resting cells caused a considerable increase in the appearance of glucose C-1 as $CO_2$, but had no significant effect with respect to glucose C-6 or oxygen uptake. Pathways for the regeneration of TPN were assessed, since the availability of the oxidized form of this pyridine nucleotide apparently determines the extent of the utilization of glucose via the direct oxidative pathway. Transhydrogenase was not found in the polymorphonuclear leukocytes of guinea pigs. $CO_2$ fixation and transhydrogenation due to the dicarboxylic acid shuttle were considered and found to be inadequate to account for the phagocytosis-induced increase in the metabolism of glucose via the direct oxidative pathway. A TPN-linked lactate dehydrogenase was found to be present in these cells and to have an adequate capacity when related to the increased conversion of glucose C-1 to $CO_2$ observed during phagocytosis.

Previous studies (Sbarra and Karnovsky, 1959) have demonstrated that a number of changes occur in the carbohydrate metabolism of phagocytizing guinea-pig polymorphonuclear leukocytes compared with those at rest. A significant stimulation of aerobic lactic acid production due to phagocytosis could be detected if a 30-minute incubation period was employed, but no stimulation was observed when measurements were made after 60 minutes. However, no significant difference in glucose consumption between resting and phagocytizing cells under aerobic conditions could be detected after 30 minutes of incubation. Under anaerobic conditions both lactic acid production and glucose consumption measured at the end of 30 minutes were increased by the process of phagocytosis (*cf.* Cohn and Morse, 1960). In addition, it was found that during phagocytosis under aerobic and anaerobic conditions the conversion of glucose C 1 to $CO_2$, measured after an arbitrarily chosen period of one hour, was greatly stimulated compared with the conversion of glucose C-6 to $CO_2$.

At present very little is known about the mechanism underlying the preferential stimulation of the oxidation of glucose C-1 to $CO_2$ during phagocytosis. In many tissues the supply of oxidized TPN appears to be one of the factors regulating the activity of the hexose monophosphate oxidative path (Kinoshita, 1957; Cahill *et al.* 1958; Brin and Yonemoto, 1958; Wenner *et al.* 1958; McLean, 1960). If TPNH oxidation were a rate-limiting factor in the operation of the direct oxidative pathway in resting leukocytes, then stimulation of TPN-regenerating reactions during phagocytosis might account for the marked increase in the oxidation of glucose C-1 compared to C-6 observed during the phagocytic process in guinea-pig leukocytes. A suggestion concerning the roles of TPN-linked lactate dehydrogenase and a DPNH-oxidase in the stimulation of conversion of glucose C-1 to $CO_2$ during phagocytosis has appeared in a preliminary communication (Evans and Karnovsky, 1961).

The studies in this paper are concerned with an examination of various aspects of aerobic carbohydrate metabolism of resting or phagocytizing leukocytes as a function of possible parameters affecting these changes, such as the time elapsed before measurements are made, the amount of glucose present, and the opportunities available to the cell for reoxidizing TPNH. Experiments will be reported on resting and phagocytizing cells incubated with glucose-1-$C^{14}$ and -6-$C^{14}$, on whole cells incubated with these labeled compounds in the presence of various electron acceptors or incubated in the presence of $C^{14}O_2$, and on subcellular fractions.

## METHODS AND MATERIALS

Suspensions rich in polymorphonuclear leu-

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kocytes (PMN) were obtained from peritoneal exudates of guinea pigs as described previously (Sbarra and Karnovsky, 1959). The quantity of the cellular material was determined by measuring the total cellular phosphorus contained in a suitable aliquot of the cell suspension prior to addition of phosphate buffer (Stähelin *et al.*, 1956a; King, 1932).

Polystyrene particles (1.171 $\mu$ in diameter) were used as the particles to be ingested and were generously supplied by Dr. J. W. Vanderhoff and the Dow Ch mical Company.

Glucose-1-$C^{14}$ and glucose-6-$C^{14}$ were obtained from the New England Nuclear Company. $BaC^{14}O$ was obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn. Enzymatically generated DPNH (87% pure) and TPNH (90% pure), DPN (95% pure), yeast alcohol dehydrogenase (twice crystallized), and phenazine methosulfate were products of the Sigma Chemical Company. Sodium pyruvate was obtained from the Nutritional Biochemical Company; acetaldehyde was a product of Matheson, Coleman and Bell, Inc. Methylene blue was a product of Allied Chemical and Dye Corporation.

## Experimental Procedures

*Respiration and Glucose Utilization.*—Respiration studies and studies involving the metabolism of glucose-1-$C^{14}$ and glucose-6-$C^{14}$ were carried out in Warburg flasks incubated at 37° with air as the gas phase and with Krebs-Ringer phosphate, pH 7.4, as the me ium. The center wells of the Warburg flasks contained a small filter paper fan and 0.2 ml of 20% KOH for absorbing $CO_2$ Cell suspensions were placed in the main compartment. The various substrates and artificial electron acceptors employed in these studies were placed in the side-arm. In experiments concerning phagocytosis a suspension of polystyrene particles (about $2 \times 10^{10}$ particles per flask) was added from the side-arm (Sbarra and Karnovsky, 1959). In all experiments the incubation medium had a final volume of 3.0 ml. The various metabolic reactions were started after a suitable equilibration period (5–10 minutes) by tipping the contents of the side-arm into the main chambers of the Warburg flasks.

The method of collecting the media for chemical analysis has been described previously (Sbarra and Karnovsky, 1959). Glucose was determined by the technique of Nelson (1944). Lactic acid analyses were carried out by the procedure of Barker and Summerson (1941).

The contents of the center wells were collected quantitatively, and the trapped $C^{14}O_2$ was precipitated as $BaCO_3$ and plated on stainless steel planchets for counting (Calvin *et al.*, 1949). The activities of the $C^{14}$-labeled glucose samples used as substrates were determined on suitable aliquots of the glucose solutions plated at infinite thinness on aluminum planchets.

*$C^{14}O_2$ Fixation Experiments.*—For studies of $CO_2$ fixation specially designed flasks which permit the generation of $C^{14}O_2$ in a closed system were employed. The flasks were approximately 10 ml. in volume and were similar in design to those used by Stähelin *et al.* (1956b). The $C^{14}O_2$ was generated from a V-shaped side-arm by tipping $HClO_4$ into $BaC^{14}O_3$ at an appropriate time. The cells were incubated in 3 ml Krebs-Ringer phosphate medium, pH 7.4. All flasks were placed on a Dubnoff apparatus and incubated with shaking at 37° for 90 minutes. At the end of the incubation period the flasks were cooled in an ice bath and the contents of the flasks were removed and analyzed as previously described (Sbarra and Karnovsky, 1959).

Lactic acid was isolated from the medium with a Dowex 1-X-1 anion exchange column (Kinoshita *et al.*, 1955). The lactic acid obtained from the ion exchange column was further purified by ascending paper chromatography in an ethanol-water-ammonia solvent system (Jones *et al.*, 1953). After drying in a stream of warm air the chromatogram was sprayed with thymol blue indicator to locate the lactate band, which could then be cut from the paper and eluted with dilute alkali. At this stage the lactate fraction contained thymol blue, so the eluted samples were run through the ion exchange column a second time. No indicator could be detected in the lactate fraction after this treatment, and the purified samples were then degraded to $CO_2$ (C-1) and acetaldehyde (C-2 and C-3) by permanganate oxidation (Brin and Olson, 1952). The $CO_2$ was trapped as $BaCO_3$ and acetaldehyde as its dimedone derivative. These compounds were plated on stainless steel planchets for counting.

$C^{14}O_2$ incorporation into cells was measured by plating suitable aliquots of washed cells on aluminum planchets and assaying for radioactivity at infinite thinness. Aliquots of the medium were also plated.

*Determination of Radioactivity.*—All radioactivity measurements were carried out with a gas flow counter in the proportional range (Robinson, 1950). Samples of barium carbonate and dimedone derivatives were counted on stainless steel planchets with a plating area of 1.54 $cm^2$ and corrected for self absorption to 5.0 mg per planchet; appropriate absorption factors were used (Karnovsky *et al.*, 1955). Radioactive measurements at infinite thinness were converted to measurements at standard thickness (5 mg per planchet) by multiplying the former by an experimentally determined conversion factor of 0.523.

*Preparation of Subcellular Fractions.*—All procedures for the homogenization and fractionation of leukocytes were carried out in a cold room at 0 to 5°. The leukocytes were suspended in about 9 volumes of ice-cold alkaline isotonic KCl (0.154 M KCl; $3.2 \times 10^{-4}$ M $KHCO_3$) (Umbreit *et al.*, 1957). The cell suspensions were homogenized for 1–2 minutes in a glass-Teflon homogenizer with a motor-driven pestle. Microscopic examination of the homogenate revealed few intact cells remaining after this procedure. The homogenate was then transferred to a graduated centrifuge tube and the total volume of the homogenate was noted. The amount of starting material could then be determined by analyzing an aliquot of the homog-

enate for total phosphorus of the disrupted cells.

All enzyme assays in this study were performed on one or the other of two fractions obtained from the whole homogenate by centrifugation. The first of these was the supernatant fraction obtained by centrifuging the whole homogenate in an International Clinical centrifuge at maximum speed for 1–2 minutes. The sediment, consisting chiefly of nuclear material, partly disrupted cells, and a few red cells, was discarded. The supernatant fraction (S-1) was very turbid and contained a considerable quantity of granules microscopically similar to those seen in intact granulocytes.

The second cell fraction used for enzyme assays was the supernatant fraction obtained by further centrifuging the S-1 fraction at 30,000 × *g* for 15 minutes in a Lourdes centrifuge. The sediment obtained was discarded. The supernatant fraction (S-2) was sometimes faintly cloudy and contained very little microscopically visible particulate matter.

All assays were usually performed on these cell fractions within 4 hours after the guinea pigs were sacrificed. For comparative purposes the amount of each fraction was determined on the basis of the amount of whole cell material, measured as total phosphorus, from which each fraction was derived.

*Spectrophotometric Enzyme Assays.*—A procedure similar to that described by Kornberg (1955) was used to determine the lactate dehydrogenase activity in the cell fractions. The standard reaction mixture for assays on the S-2 fraction consisted of the following: 0.03 M potassium phosphate buffer (pH varied as desired), 0.3 μmole of DPNH or TPNH, 1.0 μmole of pyruvate, supernatant fluid containing the enzyme (suitably diluted to give linear reaction rates) in a final volume of 3.0 ml. The reaction was initiated by the addition of pyruvate. A control reaction containing all of the reaction components except pyruvate was also included in the assay. Lactate dehydrogenase assays performed on the S-1 fraction were similar to those using the S-2 fraction except that 0.07 M phosphate buffer was employed in the reaction mixture.

Assays for transhydrogenase activity were carried out on the S-1 fraction by a procedure described by Kaplan *et al.* (1953). The various components used in the assay were as follows: TPNH 0.3 μmole, DPN 0.5 μmole, acetaldehyde 10 μmoles, DPNH 0.4 μmole, crystalline yeast alcohol dehydrogenase 1.5 μg, S-2 fraction, phosphate buffer 0.07 M (pH varied as desired) in a final volume of 3.0 ml. The control reactions used are indicated in a later section. The various reactions employed in this assay were started by adding either reduced pyridine nucleotide or alcohol dehydrogenase. In all assays the oxidation of DPNH or TPNH was determined spectrophotometrically by following the decrease in optical density at 340 mμ with a Beckman model DU spectrophotometer at room temperature (23–25°). All reactions were carried out in standard Beckman quartz cuvettes (1-cm light path). Optical density readings for assays involving the S-2 fractions were made using a water blank. For studies involving the S-1 fraction a suitably diluted portion of this fraction served as a blank for optical density measurements. Usually the change in optical density between 1.0 and 3.0 minutes was used to calculate the reaction rates, since the change in absorption was linear for this period. To convert density units into DPNH or TPNH concentration the molecular extinction coefficient of $6.22 \times 10^6$ cm$^2$ per mole (Horecker and Kornberg, 1948) was used. Measurements of pH were carried out at the end of the reaction with a Beckman model G pH meter.

## Results

*Metabolic Changes During Phagocytosis, as a Function of Time.*—In this study, glucose uptake, lactate production, oxygen uptake, and the conversion of glucose-1-$C^{14}$ and glucose-6-$C^{14}$ to $C^{14}O_2$ were measured at various time intervals in resting and phagocytizing cells. Glycogen utilization was not studied; no appreciable glycogen breakdown had previously been found in resting cells or phagocytizing cells from guinea pigs under aerobic conditions in the presence of glucose (Sbarra and Karnovsky, 1959; *cf.* Cohn and Morse, 1960). The results of these studies are shown in Table I.

The respiration of phagocytizing cells was stimulated considerably at all of the time intervals studied. Glucose utilization appeared to be stimulated initially, but this became less obvious after 5 minutes, as the total glucose consumption grew larger. Lactic acid production in phagocytizing cells was slightly greater than in resting cells up to 45 minutes of incubation, but by 90 minutes the increment was no longer significant.

In the studies involving the use of glucose-$C^{14}$ a marked increase in the conversion of glucose-1-$C^{14}$ to $C^{14}O_2$ due to phagocytosis was observed (as previously reported for an incubation period of 60 minutes) at all the time intervals studied here. Application of Student's t-test to these results indicates that this increase was highly significant after 20 minutes and after 45 minutes of incubation in these experiments. Though an increase in the conversion of glucose-6-$C^{14}$ to $C^{14}O_2$ due to phagocytosis was detected in all experiments at the 20, 45, and 90 minute intervals, the data in Table I indicate that, on the basis of four experiments, this increase was of significance only at the 20-minute interval.

The C-1:C-6 ratios for the conversion of glucose-1-$C^{14}$ and glucose-6-$C^{14}$ to $C^{14}O_2$ are also given as a function of time in Table I. During the first 20 minutes of incubation the C-1:C-6 ratio of phagocytizing cells was greater than that of resting cells. After 20 minutes, however, the C-1:C-6 ratio of phagocytizing cells tended to approach that of resting cells. In both resting and phagocytizing cells the C-1:C-6 ratio decreased after reaching a maximum at about 20 minutes.

*The Effect of Concentration on the Conversion of Glucose C-1 or C-6 to $CO_2$.*—The effect of glucose concentration on the amounts of $CO_2$ derived from glucose C-1 or C-6 was investigated with glucose labeled with $C^{14}$ in C-1 or C 6. In these experiments an incubation time of 20 minutes was em-

TABLE I

EFFECT OF PHAGOCYTOSIS ON VARIOUS METABOLIC FUNCTIONS AT DIFFERENT TIMES

| | Control Values[a] | | | | Effect of Phagocytosis (% Change) | | | |
|---|---|---|---|---|---|---|---|---|
| Time (min.) | 5 | 20 | 45 | 90 | 5 | 20 | 45 | 90 |
| Glucose used ($\mu$mole) | 0.67 ± 0.10 | 1.63 ± 0.24 | 2.61 ± 0.18 | 4.36 ± 0.28 | +23 ± 3.8 | +13 ± 6.5 | +8 ± 3.8 | −2 ± 1.9 |
| p | | | | | 0.05 | <0.10 | 0.10 | 0.20 |
| Lactate produced ($\mu$mole) | 0.88 ± 0.12 | 2.29 ± 0.09 | 4.13 ± 0.29 | 6.94 ± 0.41 | +13 ± 4.3 | +22 ± 4.0 | +20 ± 3.6 | +8 ± 2.7 |
| p | | | | | 0.03 | <0.02 | 0.02 | 0.10 |
| $O_2$ uptake ($\mu$mole) | 0.16 ± 0.02 | 0.42 ± 0.05 | 0.70 ± 0.07 | 1.07 ± 0.12 | +35 ± 8.5 | +118 ± 29 | +128 ± 34 | +131 ± 25 |
| p | | | | | <0.02 | <0.01 | <0.03 | <0.02 |
| Glucose oxidation C-1 to $CO_2$ (m$\mu$mole) | 2.46 ± 0.33 | 18.97 ± 1.29 | 45.15 ± 3.98 | 102.41 ± 8.15 | +503 ± 238 | +560 ± 88 | +632 ± 126 | +391 ± 109 |
| p | | | | | <0.2 | <0.01 | 0.01 | <0.2 |
| C-6 to $CO_2$ (m$\mu$mole) | 0.29 ± 0.07 | 0.63 ± 0.12 | 1.80 ± 0.43 | 9.54 ± 1.92 | +35 ± 24 | +273 ± 67 | +529 ± 260 | +278 ± 139 |
| p | | | | | 0.2 | <0.04 | 0.1 | 0.1 |
| Ratio C-1:C-6 | 9 | 30 | 25 | 11 | 38 | 53 | 29 | 14 |

[a] The means and standard errors of the means are given and are expressed for 100 $\mu$g cellular phosphorus. The radioactive data were corrected to an initial glucose specific activity of $1 \times 10^5$ cpm per $\mu$mole and converted to m$\mu$moles $CO_2$. Four experiments were performed. p has the usual connotation as an indication of significance. The initial glucose concentration was 5.6 mM.

ployed in order to avoid substrate exhaustion, especially in the flasks containing low concentrations of glucose, and because the data in the previous section indicated that metabolic changes due to phagocytosis were most marked at this time. The results of these experiments, shown in Table II, indicate that as the glucose concentration of the medium was increased from 1.67 mM to 16.7 mM the rates of oxidation of glucose C-1 and C-6 to $CO_2$ remained fairly constant. The oxidation of glucose C-1 to $CO_2$ was on the average about forty times greater than the oxidation of glucose C-6 to $CO_2$ under the conditions of these experiments at all of the glucose concentrations employed. It is clear that the amounts of glucose C-1 or C-6 converted to $CO_2$ are independent of glucose concentration above a value of approximately 1.7 mM. This fact makes it improbable that enhanced passage of glucose into the cells during particle ingestion is responsible for the changes observed, particularly in the oxidation of glucose C-1 to $CO_2$.

*The Effect of Added Electron Acceptors on Conversion of Glucose C-1 or C-6 to $CO_2$.*—Since glucose in the medium above a concentration of 1.7 mM apparently did not significantly limit the operation of the direct oxidative pathway for hexose monophosphate, when the conversion of glucose C-1 or C-6 to $CO_2$ was the criterion, the possibility that TPNH oxidation was a rate-limiting step in the operation of this pathway was explored using the artificial electron acceptors methylene blue and phenazine methosulfate and a physiologic electron acceptor, pyruvate (Table III). All of these compounds were found to have a pronounced stimulatory effect on the oxidation of glucose C-1 to $CO_2$ compared to the oxidation of glucose C-6. This preferential stimulation of C-1 oxidation probably was not due to an increased availability of medium glucose, since none of the added compounds caused any measurable increase in glucose uptake. Pyruvate caused some decrease in glucose utilization.

Methylene blue and phenazine methosulfate caused about a twofold and a sixfold stimulation in respiration, respectively, while added pyruvate had no significant effect. The artificial electron acceptors and pyruvate differed also in their effects on the oxidation of glucose C-6 to $CO_2$, since the former substances stimulated the conversion of glucose C-6 to $CO_2$ significantly, while pyruvate did not.

TABLE II

EFFECT OF GLUCOSE CONCENTRATION ON $C^{14}O_2$ PRODUCTION FROM GLUCOSE-1-$C^{14}$ AND GLUCOSE-6-$C^{14}$[a]

| Initial Glucose Conc. (mM) | Glucose Label | Experiment 1 Activity in $CO_2$ (cpm) | Experiment 2 Activity in $CO_2$ (cpm) | Experiment 1 C-1:C-6 | Experiment 2 C-1:C-6 |
|---|---|---|---|---|---|
| 1.67 | $C_1$ | 1068 | 3629 | 27 | 55 |
| | $C_6$ | 39 | 67 | | |
| 5.56 | $C_1$ | 1097 | 3347 | 28 | 57 |
| | $C_6$ | 39 | 58 | | |
| 16.70 | $C_1$ | 1277 | 3834 | 30 | 42 |
| | $C_6$ | 43 | 92 | | |

[a] Results are expressed on the basis of 100 $\mu$g cellular phosphorus per 20 minutes. All counts are corrected to an initial glucose specific activity of $1.0 \times 10^5$ cpm per $\mu$mole.

*TPN-Regenerating Systems in Guinea Pig Leukocytes.*—Though there are many metabolic reactions that are known to result in the regeneration of TPN from TPNH, very few of these reactions have been studied in leukocytes. The demonstration in the previous section that pyruvate preferentially stimulates the conversion of glucose C-1 to $CO_2$ suggests the possibility that such TPN-regenerating systems as the "dicarboxylic acid shuttle," TPNH-linked lactic dehydrogenase, or transhydrogenase might participate in the metabolic pattern of the leukocytes of guinea pig exudate. A study was made, therefore, to determine whether such systems are present in these cells.

The "dicarboxylic acid shuttle" might serve as a means of generating DPNH from TPNH through the coupling of TPNH-linked $CO_2$ fixation and the DPN-linked oxidation of malic acid. Studies on $CO_2$ fixation shown in Table IV indicate that $C^{14}O_2$ is incorporated into lactic acid produced by guinea pig leukocytes in the presence and absence of glucose and that the label is confined entirely to the carboxyl group of lactate. These observations

TABLE III

EFFECT OF VARIOUS ELECTRON ACCEPTORS ON RESPIRATION AND GLUCOSE UTILIZATION[a]

| | $O_2$ Uptake ($\mu$l) | Glucose Uptake ($\mu$moles) | $C^{14}O_2$[c] C-1 (cpm) | $C^{14}O_2$[c] C-6 (cpm) | Ratio C-1/C-6 |
|---|---|---|---|---|---|
| Control | 8.0 ± 0.4[b] | 1.05 ± 0.07 | 1,363 ± 410 | 13 ± 1.5 | 105 |
| Methylene blue | 15.0 ± 2.3 | 0.99 ± 0.10 | 9,871 ± 466 | 39 ± 5.5 | 253 |
| Phenazine methosulfate | 46.0 ± 2.9 | 1.08 ± 0.17 | 41,760 ± 4,445 | 79 ± 17 | 529 |
| Pyruvate | 8.0 ± 0.6 | 0.73 ± 0.12 | 3,102 ± 235 | 16 ± 3.4 | 194 |

[a] The initial glucose concentration was $5.6 \times 10^{-3}$ M. The concentration of methylene blue or phenazine methosulfate was $4.6 \times 10^{-4}$M; that of pyruvate was $9.1 \times 10^{-3}$M. [b] The means and standard errors of the means are given and are expressed on the basis of 100 $\mu$g of cellular phosphorus per 20 minutes. Three determinations were made. Radioactivity data are normalized to an initial glucose specific activity of $1.0 \times 10^5$ cpm per $\mu$mole. [c] Glucose labeled in C-1 or C-6, as substrate.

TABLE IV

FIXATION OF CARBON DIOXIDE BY GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES[a]

| Additions | Lactic acid ($\mu$moles) | Total $C^{14}O_2$ fixed: Cells (cpm) | Medium (cpm) | Lactate: $C_1$ (cpm) | Lactate: $C_2 + C_3$ (cpm) |
|---|---|---|---|---|---|
| None | 2.36 | 5,100 | 6,500 | 426 | 0 |
| Glucose (5.6 mM) | 5.42 | 10,750 | 6,200 | 2,025 | 0 |

[a] Results are expressed on the basis of 100 $\mu$g cellular phosphorus per hour. One mg $BaC^{14}O_3$ (5 $\mu$M $CO_2$; $2 \times 10^7$ cpm) served as a source of $C^{14}O_2$ in all flasks.

TABLE V

DETERMINATION OF TRANSHYDROGENASE IN THE S-1 FRACTION OF DISRUPTED LEUKOCYTES

| Additions: Pyridine nucleotide | Substrate | Alcohol Dehydrogenase | Reduced Pyridine Nucleotide Oxidized[a] ($\mu$mole): pH 6.0 | pH 7.4 |
|---|---|---|---|---|
| TPNH | | − | 0.001 | |
| TPNH | $CH_3COCOOH$ | − | 0.121 | |
| DPNH | $CH_3CHO$ | − | 0.004 | 0.005 |
| DPNH | $CH_3CHO$ | + | 0.175 | 0.164 |
| TPNH | $CH_3CHO$ | − | 0.001 | 0.004 |
| TPNH | $CH_3CHO$ | + | 0.002 | 0.002 |
| TPNH + DPN | $CH_3CHO$ | − | 0.003 | 0.005 |
| TPNH + DPN | $CH_3CHO$ | + | 0.005 | 0.005 |

[a] Results expressed as $\mu$moles per minute per 100 $\mu$g cellular phosphorus. Experimental details are given in the text. If DPNH was added to the S-1 fraction in the absence of acetaldehyde, no oxidation of DPNH could be detected.

are in accord with those of Noble *et al.* (1961), who used rabbit polymorphonuclear leukocytes. Such a labeling pattern in lactic acid is consistent with the operation of the "dicarboxylic acid shuttle." It should be noted, however, that this labeling pattern might also be explained by other mechanisms such as $C^{14}O_2$ fixation *via* ribulose-1,5-diphosphate and carboxydismutase, as suggested by Barron *et al.* (1955) for tumor cells, or by $C^{14}O_2$ fixation *via* the reversible decarboxylation of pyruvate.

To determine whether TPNH could substitute for DPNH in the conversion of pyruvate to lactate in these cells, lactic dehydrogenase assays were carried out on the S-2 fraction of disrupted cells. In Figure 1 it may be seen that dehydrogenation

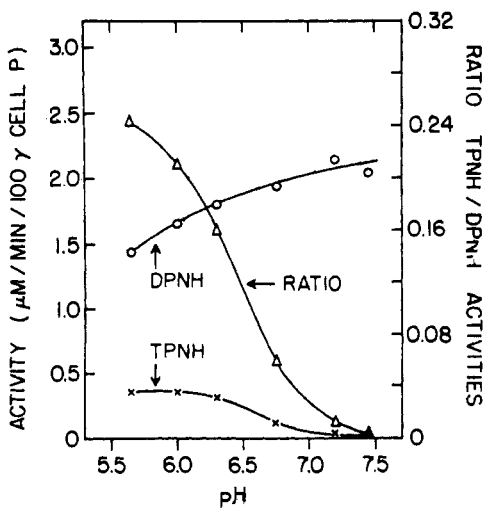


FIG. 1.—Influence of pH on DPN- and TPN-linked lactate dehydrogenase activity. Details of the assay are given under "Experimental Procedures." The ratio refers to the activity in the presence of TPNH compared with that with DPNH. Over the range studied the activity of the TPN-linked reaction increased about 35-fold and that of the DPN-linked reaction decreased about 30% as the pH was lowered.

of DPNH or TPNH in the presence of pyruvate depended markedly on pH. As pH decreased, the activity with respect to TPNH increased while that with respect to DPNH decreased. Both DPNH- and TPNH-oxidation in the absence of pyruvate were negligible.

The possibility that TPN regeneration *via* lactic dehydrogenase might be mediated by a pyridine nucleotide transhydrogenase was also explored. The test for transhydrogenase activity consisted of adding crystalline yeast alcohol dehydrogenase and acetaldehyde to the S-1 fraction in the presence of TPNH. If transhydrogenase were present, then the oxidation of TPNH would be expected to occur. Table V indicates that no transhydrogenase activity could be observed under conditions in which the addition of pyruvate to the S-1 fraction caused the rapid oxidation of TPNH. Table V also shows that, even in the presence of added DPN, no transhydrogenase activity could be detected at the two different pH values employed, and that TPNH could be significantly oxidized in the presence of pyruvate at pH 6.

## DISCUSSION

*Metabolic Rates During Phagocytosis.*—The studies on the changes in the carbohydrate metabolism of phagocytizing leukocytes at various time intervals confirm and extend previous findings (Sbarra and Karnovsky, 1959). In previous studies, however, no change in the rate of aerobic glucose utilization due to phagocytosis could be detected after 30 minutes in spite of an increase in lactic acid production. This appears to be due to the fact

that the rate of glucose utilization is stimulated during the first 5 minutes but by 20 minutes the amount consumed is not measurably different from the control level, *i.e.*, it is necessary to deal with small differences between relatively large values. The cause of the decrease in glycolysis which follows the initial increase in glycolytic rate in phagocytizing cells remains to be explored in detail. Although Sbarra and Karnovsky (1959) have postulated that a reversal of glycolysis may account for this phenomenon (*cf.* Beck, 1958a), slower rates of metabolism by cells after they have ingested particles have not been ruled out.

The marked variation of the C-1:C-6 ratios with time points up the possible differences in interpretation of experiments in which a single, arbitrarily chosen experimental period is employed. These ratios would be expected eventually to approach unity if recycling by the hexose monophosphate oxidative pathway occurred, followed by the oxidation of all the isotopic glucose carbons to $CO_2$. Marked changes in the C-1:C-6 ratios with time for the conversion of glucose-1-$C^{14}$ and glucose-6-$C^{14}$ have also been observed in tumor cells and rat heart (van Vals *et al.*, 1956).

*Regulation of the Direct Oxidation of Hexose Monophosphate.*—The results concerning the effect of the glucose in the medium on this activity indicate that the increased conversion of glucose C-1 observed during phagocytosis is probably not the result of a possible increase in the influx of glucose into the cells during phagocytosis. The data agree with the studies of Wenner *et al.* (1958) on Ehrlich ascites tumor cells. These workers employed a glucose concentration range of 0.025–10 mM. In the lactating mammary gland of the rat (McLean, 1960), however, it has been observed that the rate of oxidation of glucose-1-$C^{14}$ to $C^{14}O_2$ increased proportionally as the concentration of glucose in the medium was raised from 2 to 20 mM, while the oxidation of C-6 of glucose remained constant over this range. Cahill *et al.* (1958) have shown that the oxidation of both C-1 and C-6 of glucose by liver slices increases with increasing concentration of glucose in the medium up to about 30 mM, after which a plateau is reached.

A more likely factor in the stimulation of conversion of glucose C-1 to $CO_2$ during phagocytosis would be the increased availability of a hydrogen acceptor for TPNH regeneration to TPN. This is emphasized by the effect of electron-acceptors (Table III) on polymorphonuclear leukocytes. The preferential stimulation of conversion of glucose C-1 to $CO_2$ in the presence of added electron acceptors has also been observed in many other cells or tissues, including erythrocytes (Brin and Yonemoto, 1958), ascites tumor cells (Wenner *et al.*, 1958), and corneal epithelium (Kinoshita, 1957).

TPN-regeneration appears to regulate the hexose monophosphate oxidation path in mixed human leukocytes. In his studies on the alternate pathways of glucose-6-phosphate metabolism in homogenates of such cells, Beck (1958b) reported that, if the glucose-6-phosphate concentration is increased in the absence of added TPN, there is a large increase in the amount of glucose-6-phosphate entering the Embden-Meyerhof pathway, while no increase in the amount entering the direct oxidative pathway can be detected. As Beck suggests, an upper limit appears to be set upon the amount of glucose-6-phosphate entering the shunt, indicating that glucose-6-phosphate dehydrogenase may become saturated at lower concentrations of glucose-6-phosphate than does phosphohexose isomerase. The addition of TPN causes an increase in the amount of glucose-6-phosphate metabolized via glucose-6-phosphate dehydrogenase, however, indicating that the level of TPN may be a factor in the control of the hexose monophosphate oxidative path in human leukocytes. It was further suggested that perhaps a TPN-TPNH shuttle, existing between the dehydrogenases of that pathway and the electron transport systems connected with the tricarboxylic acid cycle, might regulate the extent of direct oxidative metabolism of glucose. No evidence was, however, offered in support of this idea. Such a TPN regenerating mechanism in guinea pig polymorphonuclear leukocytes appears unlikely at present in view of the studies of Sbarra and Karnovsky (1959). These workers have demonstrated that the operation of the hexose monophosphate oxidative pathway in intact leukocytes is little affected by concentrations of cyanide ($10^{-3}$ M) which would inhibit the electron transport system usually associated with the tricarboxylic acid cycle.

The stimulation of such TPN-regenerating systems as the TPNH-linked lactic dehydrogenase or the "dicarboxylic acid shuttle" might account for the marked stimulation of the hexose monophosphate path observed during phagocytosis. Both of these potential TPN-regenerating systems appear from the evidence obtained thus far to be present in leukocytes in guinea pig exudate. TPN regeneration via transhydrogenase appears unlikely in view of the failure to detect this enzyme in the S-1 fraction of disrupted cells. The TPN-linked lactic dehydrogenase activity appears to be a more likely candidate than the "dicarboxylic acid shuttle," since the $CO_2$ fixation data of Table IV indicate a total fixation of only about 4 m$\mu$mole $CO_2$ per hour and 100 $\mu$g cellular phosphorus, whereas the data of Table I indicate that about 120 m$\mu$mole of TPN is required for the direct oxidation of glucose C-1 to $CO_2$ by cells at rest, and about four to five times that amount for phagocytizing cells in these experiments. These values are minimal and refer only to the requirement for conversion of glucose C-1 to $CO_2$ by the direct oxidative path and do not take cycling and the conversion of C-2 and C-3, for example, to $CO_2$ into account (see below). Regeneration of TPN via TPN-linked lactic dehydrogenase at pH 5.6 may reach values as high as 18 $\mu$mole per hour and 100 $\mu$g cellular phosphorus (Fig. 1).

From the studies on the time course of metabolic changes during phagocytosis it is possible to estimate *roughly* whether the increment in lactic acid production due to phagocytosis could account for the observed increase in the activity of the direct oxidative path for hexose monophosphate, if an amount of TPN equivalent to the increment in lac-

TABLE VI
METABOLIC INCREMENTS DUE TO PHAGOCYTOSIS[a]

| | − Particles (μmoles) | + Particles (μmoles) | Increment (μmoles) |
|---|---|---|---|
| Lactic acid | 2.29 | 2.79 | 0.50 |
| $CO_2$ from glucose C-1 | $19 \times 10^{-3}$ | $125 \times 10^{-3}$ | $106 \times 10^{-3}$ |
| $CO_2$ from glucose C-6 | $0.63 \times 10^{-3}$ | $2.40 \times 10^{-3}$ | $1.8 \times 10^{-3}$ |

[a] All values expressed as μmoles per 20 minutes per 100 μg cellular phosphorus.

tic acid production during phagocytosis were regenerated via lactic dehydrogenase. For convenience the data necessary for this estimate are shown in Table VI. These data have been derived from Table I, and all rates have been calculated from the metabolic changes observed during the first 20 minutes of incubation. It is obvious from the data in Table VI that 0.5 μmole of TPN could be regenerated as a result of the increased conversion of pyruvate to lactate during phagocytosis. Using the difference between the increments in the conversion of glucose C-1 and glucose C-6 to $CO_2$ as a measure of the increase in the activity of the direct oxidation of hexose monophosphate it may be calculated that about 0.10 μmole of $CO_2$ is produced from glucose C-1 as a result of increased activity of the direct oxidative pathway during phagocytosis. Since 2 μmoles of TPN are required to produce 1 μmole of $CO_2$ *via* that pathway, about 0.20 μmole of TPN would have to be regenerated in order to account for the increased conversion of glucose C-1 to $CO_2$. This amount of TPN is only about 40% of the estimated amount of TPN that might maximally be provided by TPNH-linked production of the extra lactate due to phagocytosis, or about 8% of the total lactate. It must be borne in mind, however, that the specific activity data used as a basis for this calculation are those of the substrate glucose. It was found, using the method of Kemp and van Heijningen (1954), that the specific activity of the intracellular glucose was indeed identical to that of the added glucose. The specific activity of the glucose-6-phosphate pool, which would be more relevant, is not known but could be lower than that of substrate glucose if some glycogen glucose were participating. That the latter amount is small is indicated by the fact that the specific activity of lactate carbon produced in the presence of uniformly labeled glucose is depressed by only 20% due to lactate production from other sources. That glucose from glycogen yields but little lactate is also suggested by the results of Noble *et al.* (1961), who demonstrated that in rabbit polymorphonuclear leukocytes $C^{14}O_2$ fixation yielded glycogen-glucose with about 40% of its activity in C-1 and 60% in C-6, whereas lactate was labeled only in C-1. If recycling occurs in the direct oxidative pathway for glucose-6-phosphate in guinea pig polymorphonuclear leukocytes, a portion of the remaining potentially available TPN regenerated *via* lactic dehydrogenase (60%) might be used. In an experiment with glucose-2-$C^{14}$, for example, it was found that in 2 hours one quarter as much C-2 appeared as $CO_2$ as did C-1. The C-1:C-6 ratio at that time in that experiment was 12.

Provided that the assumptions given above are valid it may be concluded from these rough calculations that the observed increase in lactate production is sufficient to account for the increase in activity of the hexose monophosphate shunt during phagocytosis.

The effect of pH on TPNH-linked and DPNH-linked lactic dehydrogenase activities observed here is similar to that found in other tissues such as rat liver (Navazio *et al.*, 1957), rat brain, Novikoff tumor (Potter and Niemeyer, 1959), and cattle retina (Futterman and Kinoshita, 1959). The TPNH-linked lactic dehydrogenase could be active in the intact cell, even though a low pH is required for maximal activity. Ishilawa (1935) has estimated the pH in the cytoplasm of intact polymorphonuclear leukocytes to be 6.6. pH values well on the acid side in the vicinity of the granules of peritoneal exudate cells have been reported by Rous (1925). Increased glycolytic activity during phagocytosis might result in even lower values and might thus regulate the relative amounts of DPNH and TPNH utilized for the reduction of pyruvate.

The participation of other possible TPN-regenerating systems in the stimulation of direct oxidative activity during phagocytosis is not ruled out. The point that the hexose monophosphate oxidative path may function to provide TPNH for specific reductive synthetic processes has often been made and is particularly applicable to lipid synthesis. The possibility that increased lipid synthesis might occur in leukocytes during phagocytosis has been investigated by Elsbach (1959), by Sbarra and Karnovsky (1960), and by Karnovsky and Wallach (1961). An increased incorporation of radioactive precursors into the lipid fractions of phagocytizing cells was demonstrated. It was not possible to detect net synthesis of lipid, and it is not clear yet whether the increased labeling of the lipids can be correlated with the stimulation of conversion of glucose C-1 to $CO_2$ during particle ingestion.

In view of the data obtained here, and those concerning an active cyanide-insensitive DPNH-oxidase present in polymorphonuclear leukocytes (Evans and Karnovsky, 1961), it is postulated that the TPN-linked lactic dehydrogenase plays an important, perhaps dominant, role in stimulating the hexose monophosphate oxidative path during phagocytosis.

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# Myoinositol Polyphosphate Intermediates in the Dephosphorylation of Phytic Acid by Phytase*

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Phytic acid, on partial dephosphorylation with phytase, yields a series of optically active polyphosphate esters which heretofore had not been characterized. These degradation products have now been identified as myoinositol 1- and 2-monophosphate; myoinositol 1,2-diphosphate; myoinositol 1,2,3- and L-1,2,6-triphosphate; myoinositol 1,2,5,6-tetraphosphate plus another uncharacterized isomer. The structure of the myoinositol pentaphosphate has not been established. Some of the apparent specificities of phytase indicated by this series of characterized isomers are discussed.

Posternak and Posternak (1929) reported that optically active myoinositol polyphosphate esters were produced by the partial dephosphorylation of phytic acid by the enzyme "phytase." The enzyme preparation used in these experiments was an unfractionated aqueous extract of wheat bran. The products of the reaction were isolated as barium salts by fractional precipitation, and their characterization as myoinositol di-, tri-, and tetraphosphates was based on elemental analysis.

Subsequently, Courtois and co-workers carried out an extensive investigation on this reaction, the results of which have been summarized in a review article (Courtois, 1951). By fractionation of extracts from a variety of plant sources, they obtained in all cases two phosphatase components. One of these, designated as the phosphomonoesterase fraction, showed no phosphatase activity on phytic acid but was active on glycerol 2-phosphate. The second component, called the phytophosphatase fraction, was active in dephosphorylating phytic acid and myoinositol polyphosphate esters, but was also equal to or better than the phosphomonoesterase in dephosphorylating glycerol 2-phosphate. No enzyme fraction was obtained that possessed greater phosphatase activity on phytic acid than on simple phosphate esters. In all, the studies failed to demonstrate an enzyme specific for the dephosphorylation of phytic acid.

The nature of the myoinositol polyphosphate products of the reaction was also investigated by Courtois (1951). The studies involved a comparison of the rates of oxidation of myoinositol hexa-, penta-, tetra-, and triphosphate to the rates of oxidation of reference compounds. The oxidants employed were bromine in bicarbonate solution, potassium bichromate in nitric acid, and sodium periodate in sulfuric acid. The myoinositol polyphosphate esters either were not oxidized or, in the case of tri- and tetraphosphates, were oxidized at a rate much slower than the reference compounds. This behavior was interpreted as indicating the absence of vicinal hydroxyl groups in these esters. Therefore, Courtois proposed that the enzymatic dephosphorylation of phytic acid proceeded as follows.

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