REFERENCES

- Adams, J. T., Abramovitch, B. & Hauser, C. R. (1943). *J. Amer. chem. Soc.* **65**, 552.
- Alexander, E. R. & Cope, A. C. (1944). *J. Amer. chem. Soc.* **66**, 886.
- Bendz, G. (1959). *Ark. Kemi*, **14**, 511.
- Benjamin, C. R. & Stodola, F. H. (1960). *Nature, Lond.*, **188**, 662.
- Bowman, R. E. (1950). *J. chem. Soc.* p. 322.
- Brewer, D. (1958). *Canad. J. Bot.* **36**, 941.
- Eistert, B. & Reiss, W. (1954). *Chem. Ber.* **87**, 92.
- Hendershot, W. F., Hesseltine, C. W., Pridham, T. G., Benedict, R. G. & Jackson, R. W. (1962). *Arch. Biochem. Biophys.* **96**, 166.
- Hesseltine, C. W., Benjamin, C. R., Bradle, B. & Hendershot, W. F. (1963). *Amer. J. Bot.* **50**, 209.
- Hückel, W., Neunhoeffer, O., Gercke, A. & Frank, E. (1930). *Liebigs Ann.* **477**, 130.
- Ives, D. J. G. & Sames, K. (1943). *J. chem. Soc.* p. 513.
- Kögl, F. & DeBruin, O. A. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 729.
- Neill, K. G. & Raistrick, H. (1957). *Biochem. J.* **65**, 166.
- Nishikawa, E. (1933a). *J. agric. chem. Soc. Japan*, **9**, 772.
- Nishikawa, E. (1933b). *J. agric. chem. Soc. Japan*, **9**, 1073.
- Rasmussen, R. S., Tunnicliffe, D. D. & Brattain, R. R. (1949). *J. Amer. chem. Soc.* **71**, 1068.
- Sondheimer, E. (1957). *J. Amer. chem. Soc.* **79**, 5036.
- Stout, G. H., Dreyer, D. L. & Jensen, L. H. (1961). *Chem. & Ind.* p. 289.
- Venus-Danilowa, E. (1928). *Ber. dtsh. chem. Ges.* **61**, 1954.
- Yabuta, T. & Sumiki, Y. (1933). *J. agric. chem. Soc. Japan*, **9**, 1264.
- Yamamoto, I., Nitta, K. & Yamamoto, Y. (1961). *Agric. biol. Chem., Tokyo*, **25**, 405.
- Yamamoto, I., Nitta, K. & Yamamoto, Y. (1962). *Agric. biol. Chem., Tokyo*, **26**, 486.
- Yates, P. & Stout, G. H. (1958). *J. Amer. chem. Soc.* **80**, 1691.

Biochem. J. (1964), **93**, 97

Effects of Insulin and Starvation on the Metabolism of Acetate and Pyruvate by the Perfused Rat Heart

By J. R. WILLIAMSON

Department of Biochemistry, University of Oxford, and the Baker Clinic Research Laboratory, New England Deaconess Hospital, in the Department of Medicine, Harvard Medical School, Boston, Mass., U.S.A.

(Received 13 December 1963)

Studies of myocardial metabolism *in vivo* by means of catheterization of the coronary sinus have revealed the prominent role of fatty acids and ketone bodies as metabolic fuels for energy production in the intact human and dog heart (Bing, 1954–55). Extraction of carbohydrates has been shown to be predominant only in the post-absorptive stage after a high-carbohydrate meal, and to be greatly decreased in the starved and diabetic states (Olson & Piatnek, 1959; Goodale, Olson & Hackel, 1959). Investigations of the metabolism of the isolated perfused rat heart have likewise shown that ketone bodies (Williamson & Krebs, 1961) or fatty acids (Shipp, Opie & Challoner, 1961) are readily utilized as respiratory fuels when supplied to the heart as the sole substrate. Lactate and glucose, on the other hand, are relatively poorly utilized by hearts from normal fed rats, even in the presence of insulin (Williamson, 1962a). When mixtures of substrates are added to the perfusion fluid, glucose competes unfavourably with fatty acids and ketone bodies for oxidation, and tends to be metabolized by alternative pathways to lactate and glycogen.

Hearts from starved rats demonstrate a decreased utilization of glucose and lactate, but aceto-

acetate utilization is unchanged (Williamson & Krebs, 1961; Williamson, 1962a). An impaired utilization of glucose and pyruvate by muscle tissues from diabetic rats has been observed by many groups of investigators (Krahl & Cori, 1947; Pearson, Hsieh, Dutoit & Hastings, 1949; Vilee & Hastings, 1949; Kipnis, Helmreich & Cori, 1959; Morgan, Cadenas, Regen & Park, 1961), and a decreased rate of pyruvate decarboxylation has been suggested as a factor contributing to the abnormal response of diabetic subjects to glucose and pyruvate tolerance loads (Fry & Butterfield, 1962). Insulin has generally not been observed to restore fully the rate of glucose phosphorylation by muscle from diabetic animals, but has been reported to increase to normal the oxidation of pyruvate by diaphragms from alloxan-diabetic rats (Pearson *et al.* 1949; Vilee & Hastings, 1949). These studies suggest that insulin may have a permissive role in the rate of pyruvate entry into the citric acid cycle in muscle from diabetic animals.

The present investigation is concerned with a study of the effect of insulin on pyruvate and acetate utilization by perfused hearts from fed and starved rats and the metabolic competitions

exhibited between these substrates and glucose. Preliminary accounts of this work have been published (Williamson, 1961, 1962*b*).

METHODS

The perfusion apparatus and experimental techniques used for the non-isotopic experiments were similar to those described by Williamson & Krebs (1961). Male rats (Wistar strain) weighing between 220 and 280 g. were used. 'Well-fed' animals had unrestricted access to food and water, and starved rats were deprived of food for 24 hr. before being killed. Other rats were maintained on a high-carbohydrate diet for at least 1 week (Williamson, 1962*a*). The initial volume of the perfusate was usually 20 ml., and 1 ml. samples were removed for analysis after 15 and 75 min. In a few experiments a larger volume of perfusate was used and samples were removed every 10 or 15 min. The perfusion temperature in all experiments was 37°.

Isotope measurements. Approx. 5 μ C of [2-¹⁴C]acetate/25 ml. of perfusate was used, and the initial acetate concentration was 10 mM. The normal perfusion apparatus was modified so that the gas outlet was connected to a CO₂-absorption unit containing 4 ml. of 2 N-NaOH. This unit was changed every 15 min. Duplicate samples of perfusate were taken at 15 min. intervals for the chemical determination of acetate and the counts present as [¹⁴C]bicarbonate. The perfusate sample containing [¹⁴C]bicarbonate was diluted fourfold with 0.1 M-NaHCO₃, and the CO₂ from duplicate samples was volatilized into 2 N-NaOH by the addition of acid. Control experiments showed that [¹⁴C]-acetate did not diffuse into the NaOH. The ¹⁴C in the NaOH solutions was precipitated as BaCO₃ with BaCl₂ (5%, w/v) and plated in duplicate on paper disks (2.52 cm.²) as described by Sakami (1955). The samples were assayed for radioactivity in a gas-flow counter (Nuclear-Chicago Corp. model D-47), and the counts were corrected for self-absorption. The total number of counts collected was generally 5000, and the error in plating duplicate samples was within $\pm 2\%$. The total metabolic ¹⁴CO₂ was obtained from the sum of the counts appearing as CO₂ in the effluent gas, and those appearing in the medium bicarbonate. These counts were converted into μ moles of acetate equivalents by dividing by the specific activity of [¹⁴C]acetate, which was within the range 7000–9000 counts/min./ μ mole for different experiments. The specific activity of [2-¹⁴C]acetate was obtained by total oxidation of a sample of the initial perfusion medium with Van Slyke–Folch reagent as described by Sakami (1955).

The ¹⁴C incorporated into lipid and fatty acids was obtained by saponifying, with hot KOH (30%, w/v), samples

of the heart after perfusion, followed by extraction with light petroleum as described by Winegrad & Renold (1958). A portion of the light-petroleum extract was evaporated to dryness and oxidized to CO₂ with Van Slyke–Folch reagent.

Analytical methods. Acetic acid was liberated from acetate in the perfusate (up to 10 μ moles/ml.) by the micro-diffusion method of Serlin & Cotzias (1952), with Conway units 6.5 cm. in diameter, and titrated against 0.02 N-NaOH in an atmosphere of N₂ by using an automatic titrator (Radiometer, Copenhagen). Pyruvate was estimated in a 1:25 dilution of the perfusate by the enzyme method of Holzer & Holldorf (1957). Glucose, glycogen, lactate and oxygen consumption were measured by the methods used by Williamson & Krebs (1961). Glycogen values are expressed as glucose equivalents.

The extracellular space of the perfused heart was determined with raffinose (5 mg./ml.), which was estimated by the method used for inulin by Fisher & Lindsay (1956). The perfusate was diluted 1:100 for raffinose estimation. For determination of the acetate and raffinose in the heart, a weighed heart sample was homogenized in 2 ml. of 0.6 N-perchloric acid and centrifuged, and the precipitate was washed with 1 ml. of water. The combined supernatants were neutralized with 2 N-KOH, made up to 5 ml. and centrifuged. A 2 ml. sample of the supernatant, diluted to 20 ml., was used for raffinose estimation, the undiluted supernatant was used for acetate analysis, and the acetic acid was titrated against 5 mM-NaOH. The acetate and raffinose spaces were calculated according to the formulae of Morgan, Henderson, Regen & Park (1961). An acetate space greater than the raffinose space denotes intracellular accumulation of acetate.

Samples of the neutralized perchloric acid extract of the heart after perfusion with 10 mM-[2-¹⁴C]acetate were chromatographed two-dimensionally on paper and radioactive compounds were located by radioautography, as described by Large, Peel & Quale (1961). Glutamic acid was analysed quantitatively on a one-dimensional chromatogram by comparison of the heart extract with standard glutamic acid solutions, by using the method described by Hicks & Kerly (1960). Citrate was determined according to the method of Taylor (1953).

RESULTS

Rate of uptake and oxidation of acetate. The rat heart rapidly removed acetate from the perfusion medium. Table 1 shows an experiment in which the initial acetate concentration was 5 mM. The rate of acetate uptake remained approximately constant

Table 1. *Rate of acetate uptake by the perfused rat heart*

The heart from a well-fed rat (132 mg. dry wt.) was perfused with 25 ml. of medium containing sodium acetate (initial concn. 5 mM). Samples (1 ml.) were removed for analysis at the times stated.

Time (min.)	0	15	30	45	60	75	90	105
Concn. of acetate (mM)	5.00	4.31	3.25	2.25	1.25	0.47	0.13	0.00
Acetate uptake (μ moles)	—	16.6	41.0	63.0	84.0	99.6	106.1	108.4
Rate of acetate uptake over consecutive 15 min. intervals (μ moles/g. dry wt./hr.)	—	504	740	668	637	473	107	70
Contribution of acetate to the fuel of respiration (%)	—	64	87	79	75	56	23	8

and was about $640 \mu\text{moles/g. dry wt./hr.}$ for the first 60 min. of the experiment, during which time the acetate concentration fell to 1.25 mM . Below this concentration, the rate of acetate uptake rapidly declined until all the acetate was removed from the medium. Measurement of the oxygen consumption of four hearts perfused with acetate gave a mean value of $1735 \pm 52 \mu\text{moles/g. dry wt./hr.}$, which is close to the values obtained by Fisher & Williamson (1961) for hearts perfused with glucose. Since it was impracticable to measure the oxygen consumption of hearts at the same time as the acetate uptake, the value of $1700 \mu\text{moles/g. dry wt./hr.}$ has been used to calculate the contribution of acetate to the fuel of respiration. Over the first 60 min. of the experiment shown in Table 1, the complete oxidation of the acetate which was removed from the medium accounted on the average for 75 % of the oxygen consumption. After 75 min., when the concentration of acetate had fallen below 0.5 mM , acetate removal accounted for 23 % or less of the total respiration. Other experiments starting with 10 mM -acetate showed that the rate of acetate uptake remained linear for at least 90 min., and this concentration was used in the subsequent experiments.

The assumption that acetate disappearance from the medium was equivalent to acetate oxidation was tested with $[2-^{14}\text{C}]$ acetate. The total production of $^{14}\text{CO}_2$ was obtained from the sum of the radioactivity appearing in the medium bicarbonate and that evolved as carbon dioxide, both of which were measured over successive 15 min. intervals. The results of one such experiment where insulin ($2 \text{ million units/ml.}$) was present in addition to $[2-^{14}\text{C}]$ -acetate (10 mM) are shown in Fig. 1. The acetate uptake remained approximately linear during the 2 hr. perfusion period at a mean rate of $700 \mu\text{moles/g. dry wt./hr.}$, and the concentration of acetate in the medium fell to 3.5 mM . There was a lag in the production of $^{14}\text{CO}_2$ over the first 30 min. of the experiment, but thereafter 78–94 % of the counts removed from the medium were recovered as carbon dioxide, showing that after the initial lag period most of the acetate removed by the heart was completely oxidized. A similar experiment to that shown in Fig. 1 in which a heart was perfused with 10 mM - $[2-^{14}\text{C}]$ acetate but no insulin showed that 77 % of the acetate disappearing was converted into $^{14}\text{CO}_2$ over the period of perfusion from 30 to 120 min., and the average rate of acetate uptake was $640 \mu\text{moles/g. dry wt./hr.}$

The incorporation of ^{14}C into lipid and fatty acids was less than $0.1 \mu\text{mole/g. dry wt.}$ (four hearts) both in the presence and absence of insulin. After a 20 min. perfusion period, analysis of the heart extract showed that $2.0 \mu\text{moles}$ of $[2-^{14}\text{C}]$ acetate were converted into glutamic acid and $0.32 \mu\text{mole}$

into citric acid. Radioactive spots corresponding to other labelled compounds were not identified on the radioautogram. The total glutamic acid content of the heart (dry wt. 154 mg.) was $4.5 \mu\text{moles}$ and the citric acid content was $0.4 \mu\text{mole}$.

Effects of insulin and nutritional state on the metabolism of acetate. Insulin produced a 20 % increase of acetate uptake in hearts from both fed rats and those starved for 24 hr. (Table 2). This effect was statistically significant ($P < 0.01$). Lactate formation was small and was unaffected by insulin. Glycogen changes during perfusion were small. The contribution of acetate to the total fuel of respiration was increased by insulin from 74–77 to 91–94 %.

The intracellular concentration of acetate was measured in separate experiments, in which raffinose (5 mg./ml.) was used to measure the extracellular space. Insulin did not affect the total water content or the raffinose space of the hearts, but the acetate space and intracellular acetate concentration were decreased. The ratio of intracellular to the extracellular acetate concentrations after 30 min. of perfusion was decreased by insulin from 84 ± 4.5 to 60 ± 5.4 % (Table 3).

Starvation or a high-carbohydrate diet had no significant effect on the uptake of acetate, formation of lactate or the glycogen content of hearts during perfusion (Table 2), although glycogen concentrations in hearts from starved rats after 15 min. were elevated 80 % above the fed controls.

Rate of utilization of pyruvate at low concentrations. An experiment in which pyruvate (1 mM) was the substrate added to the perfusate is shown in Fig. 2. Over the first 40 min. of perfusion, pyruvate was

Fig. 1. Rate of $[2-^{14}\text{C}]$ acetate uptake and oxidation by perfused rat heart. $\circ$, Acetate uptake (μmoles); $\triangle$, $^{14}\text{CO}_2$ formation (μmoles of acetate equivalents); $\bullet$, concn. of acetate (mM).

Table 2. *Effects of insulin and diet on the utilization of acetate by the perfused rat heart*

Rat hearts were perfused with 20 ml. of medium containing sodium acetate (initial concn. 10 mM). Samples of perfusate were removed for analysis after 15 and 75 min. Values shown are means $\pm$ S.E.M.

Nutritional state	Insulin (milliunits/ ml.)	No. of expts.	Metabolic changes (μ moles/g. dry wt./hr.)					Contribution of acetate to fuel of respiration (%)
			Acetate uptake	Lactate formation	Glycogen			
					Found at 15 min.	Found at 75 min.	Difference	
Well-fed	0	6	633 $\pm$ 19	38 $\pm$ 7	77 $\pm$ 6	81 $\pm$ 5	+4 $\pm$ 8	74
	2	6	773 $\pm$ 21	9 $\pm$ 4	69 $\pm$ 7	83 $\pm$ 6	+14 $\pm$ 9	91
Starved for 24 hr.	0	6	655 $\pm$ 22	26 $\pm$ 2	127 $\pm$ 10	115 $\pm$ 12	-12 $\pm$ 16	77
	2	6	798 $\pm$ 38	30 $\pm$ 10	120 $\pm$ 5	107 $\pm$ 12	-13 $\pm$ 12	94
High-carbohydrate diet	0	4	595 $\pm$ 31	23 $\pm$ 3	65 $\pm$ 7	50 $\pm$ 10	-15 $\pm$ 12	70

Table 3. *Effect of insulin on the uptake and intracellular concentration of acetate in the perfused rat heart*

Hearts from well-fed rats were perfused for 30 min. with 20 ml. of medium containing sodium acetate (initial concn. 10 mM). Values shown are means $\pm$ S.E.M.

Insulin (milliunits/ ml.)	No. of hearts	Acetate uptake (μ moles/g. dry wt./30 min.)	Total water (μ l./g. dry wt.)	Raffinose space (μ l./g. dry wt.)	Acetate space (μ l./g. dry wt.)	Intracellular acetate extracellular acetate concentration ratio (%)
0	8	321 $\pm$ 11	4410 $\pm$ 52	1903 $\pm$ 42	4010 $\pm$ 93	84 $\pm$ 4.5
2	8	374 $\pm$ 9*	4490 $\pm$ 48	1960 $\pm$ 52	3470 $\pm$ 161†	60 $\pm$ 5.4*

* Significance of difference from control: $P < 0.01$.

† Significance of difference from control: $0.02 > P > 0.01$.

Fig. 2. Rate of pyruvate uptake and lactate formation at low pyruvate concentrations. $\circ$, Pyruvate uptake (μ moles); $\triangle$, lactate formation (μ moles); $\bullet$, concn. of pyruvate (mM); $\blacktriangle$, concn. of lactate (mM).

rapidly removed from the medium at an approximately constant rate of 550 μ moles/g. dry wt./hr., and the perfusate pyruvate concentration fell to 0.2 mM. Below this concentration, the rate of pyruvate uptake fell progressively until all the

pyruvate was metabolized. Some lactate was formed during the first 40 min. and this was subsequently utilized by the heart. At concentrations above 0.2 mM, the rate of pyruvate uptake was sufficient to account for about 65 % of the total oxidative metabolism, whereas at concentrations below 0.1 mM it accounted for only 6–12 %. When the pyruvate concentration fell below 0.1 mM, lactate disappearance was sufficient to contribute 14–20 % to the metabolic fuel. During the phase of net lactate utilization (after 40 min. of perfusion), the lactate:pyruvate concentration ratio in the perfusate increased from 1.2 to 3.1.

Utilization of pyruvate at high concentrations. Similar experiments to that shown in Fig. 2, but with an initial pyruvate concentration of 10 mM, showed that the rate of pyruvate uptake and formation of lactate were constant over 75 min. of perfusion, and this concentration was used in subsequent experiments. With fed rats the mean rate of pyruvate uptake was 845 μ moles/g. dry wt./hr., and approx. 30 % of the pyruvate disappearing from the medium was converted into lactate (Table 4). Glycogen changes during perfusion were small, showing that glycogen was not the precursor of medium lactate. Under these circumstances, pyruvate contributed about 90 % to the fuel of respiration. This value was calculated by multiplying the pyruvate uptake after subtraction of the lactate formation by 2.5 (the number of molecules

of oxygen needed to oxidize one molecule of pyruvate) and dividing by the oxygen consumption. This was assumed to be 1700 μ moles/g. dry wt./hr. This calculation further assumes that the pyruvate removed from the medium and not converted into lactate was completely oxidized. The contribution of the reducing equivalents needed for lactate formation is also neglected, and hence the values given probably represent an upper limit for the true values.

Effect of starvation and insulin on pyruvate utilization. Starvation (for 24 hr.) significantly decreased the pyruvate uptake from 845 ± 34 to 751 ± 16 μ moles/g. dry wt./hr. ($P < 0.01$), whereas lactate formation was not significantly changed. A similar effect was seen with rats starved for 48 hr. The proportion of pyruvate converted into lactate was increased from 28 to 35 % by starvation (24 or 48 hr.), and the contribution of pyruvate to the fuel of respiration was decreased from 90 to 71 % (Table 4). Insulin had no effect on the utilization of pyruvate by hearts from fed rats, but it increased the pyruvate uptake by hearts from rats starved for 24 to 48 hr. to values similar to those observed with fed rats, and the contribution of pyruvate to the fuel of respiration was increased from 71 to 80–86 %. Significant glycogen changes from 15 to 75 min. of perfusion were not observed in hearts from either fed or starved rats.

Competition for metabolism between acetate and pyruvate. When acetate and pyruvate were added to the perfusate at an initial concentration of 10 mM, both substrates were removed from the medium (Table 5). Each substrate competed with the other for utilization, so that the concentration of acetate to the fuel of respiration was decreased from 74 to 35 % by the additional presence of pyruvate, and the contribution of pyruvate was decreased from 90 to 60 % by the additional presence of acetate. The proportion of the pyruvate uptake appearing as lactate in the medium was increased from 28 to 40 % in the presence of acetate. Glycogen changes in the hearts during perfusion were not observed.

Competition for metabolism between glucose, acetate and pyruvate in the presence of insulin. The competition for metabolism in hearts from fed rats between glucose on the one hand and acetate or pyruvate on the other hand was studied by adding either acetate (10 mM) or pyruvate (10 mM) to medium containing glucose (5 mM) and insulin (2 milliunits/ml.). Acetate and pyruvate decreased glucose uptake by 40 and 45 % respectively (Table 6). The presence of acetate caused an increase in lactate formation from 114 ± 14 to 162 ± 31 μ moles/g. dry wt./hr. Most of this lactate must have been formed from glucose since lactate formation in the presence of acetate alone was small (Table 2). The amount of

Table 4. *Effects of insulin and starvation on the utilization of pyruvate by the perfused rat heart*

Rat hearts were perfused with 20 ml. of medium containing sodium pyruvate (initial concn. 10 mM). Samples (1 ml.) were removed for analysis after 15 and 75 min. The values shown are means $\pm$ S.E.M.

Metabolic changes (μ moles/g. dry wt./hr.)									
Nutritional state	Insulin (milli-units/ml.)	No. of hearts	Pyruvate uptake	Lactate formation	Glycogen			Pyruvate not accounted for as lactate	Contribution of pyruvate to fuel of respiration (%)
					Found at 15 min.	Found at 75 min.	Difference		
Well-fed	0	6	845 $\pm$ 34	238 $\pm$ 19	100 $\pm$ 11	89 $\pm$ 4	- 11 $\pm$ 12	607 $\pm$ 31	90
	2	6	851 $\pm$ 13	255 $\pm$ 34	69 $\pm$ 7	83 $\pm$ 7	+ 14 $\pm$ 10	596 $\pm$ 29	88
Starved for 24 hr.	0	6	751 $\pm$ 16	263 $\pm$ 17	126 $\pm$ 12	126 $\pm$ 6	0	488 $\pm$ 29	71
	2	6	846 $\pm$ 25	260 $\pm$ 22	109 $\pm$ 6	132 $\pm$ 14	+ 23 $\pm$ 17	586 $\pm$ 11	86
Starved for 48 hr.	0	8	750 $\pm$ 14	267 $\pm$ 22	154 $\pm$ 5	157 $\pm$ 3	+ 3 $\pm$ 6	484 $\pm$ 34	71
	2	8	820 $\pm$ 26	274 $\pm$ 15	140 $\pm$ 5	136 $\pm$ 9	- 6 $\pm$ 12	546 $\pm$ 25	80

Table 5. *Competition for metabolism between acetate and pyruvate in the perfused rat heart*

Hearts from well-fed rats were used and the initial volume of perfusate was 20 ml. Samples of perfusate were removed for analysis after 15 and 75 min. The values shown are means $\pm$ S.E.M.

Additions to medium (initial concn.)		No. of hearts	Metabolic changes (μ moles/g. dry wt./hr.)			Contribution to fuel of respiration (%)	
Acetate (mM)	Pyruvate (mM)		Acetate uptake	Pyruvate uptake	Lactate formation	Acetate	Pyruvate
10	0	8	633 ± 19	—	38 ± 7	74	—
0	10	6	—	845 ± 34	238 ± 19	—	90
10	10	5	299 ± 10	670 ± 27	265 ± 27	35	60

Table 6. *Competition for metabolism between glucose, acetate and pyruvate in the presence of insulin*

Hearts from well-fed rats were used and the initial volume of perfusate was 20 ml. Values shown are means $\pm$ S.E.M.

Additions to medium (initial concn.)			Insulin (milli- units/ ml.)	No. of hearts	Metabolic changes (μ moles/g. dry wt./hr.)						
Glucose (mm)	Acetate (mm)	Pyr- uvate (mm)			Acetate uptake	Pyruvate uptake	Glucose uptake	Lactate formation	Found at 15 min.	Found at 75 min.	Difference
5	0	0	2	6	—	—	266 $\pm$ 7	114 $\pm$ 14	94 $\pm$ 6	140 $\pm$ 9	+ 45 $\pm$ 11
5	10	0	2	6	660 $\pm$ 12	—	159 $\pm$ 14	162 $\pm$ 31	91 $\pm$ 6	154 $\pm$ 5	+ 63 $\pm$ 8
5	0	10	2	8	—	761 $\pm$ 28	147 $\pm$ 7	224 $\pm$ 14	72 $\pm$ 7	161 $\pm$ 8	+ 89 $\pm$ 12

lactate formed from glucose in the presence of pyruvate could not be ascertained since lactate was also formed from pyruvate. Glycogen synthesis was increased from 45 ± 11 to $63 \pm 8 \mu$ moles/g. dry wt./hr. by acetate, and from 45 ± 11 to $89 \pm 12 \mu$ moles/g. dry wt./hr. by pyruvate (Table 6).

Glucose with insulin decreased the acetate uptake from 773 ± 21 to $660 \pm 12 \mu$ moles/g. dry wt./hr. and decreased the pyruvate uptake from 851 ± 13 to $761 \pm 28 \mu$ moles/g. dry wt./hr. These changes, though small, were statistically significant ($P < 0.01$). Even in the presence of glucose and insulin, acetate oxidation accounted for 78 % of the estimated oxygen consumption, whereas glucose oxidation accounted for only 5 %. It was not possible to evaluate separately the contributions of glucose and pyruvate to the total respiration, but pyruvate oxidation clearly predominated over glucose oxidation, and taken together their respiration accounted for all the oxygen consumption.

DISCUSSION

Acetate and pyruvate as respiratory fuels. Acetate was removed from the medium of the perfused rat heart at a constant rate over the concentration range 1–10 mM, and the acetate disappearance was sufficient to account for about 75 % of the total fuel of respiration. This value is similar to the values of 73–82 % obtained for the contribution of acetoacetate to the fuel of respiration in the perfused rat heart (Williamson & Krebs, 1961). The rate of acetate uptake was dependent on concentration below 1 mM, but even at concentrations of 0.1–0.5 mM acetate accounted for 20 % of the respiratory fuel. Most of the acetate disappearing from the medium was directly oxidized since after an initial 15–30 min. lag period 80–90 % of the radioactivity of the $[2-^{14}\text{C}]$ acetate taken up was recovered in the carbon dioxide. Conversion of $[2-^{14}\text{C}]$ acetate into long-chain fatty acids was observed to be very small, although Hülsmann (1960) has reported that such synthesis can occur in heart sarcosomes. Other experiments have shown that the incorporation of

^{14}C from $[2-^{14}\text{C}]$ acetate into heart glycogen was also negligible (Kreisberg & Williamson, 1964). Lundquist (1962) has shown that blood acetate concentrations were increased after ethanol ingestion by human subjects, and the rapid oxidation of acetate by cardiac muscle is in general agreement with the findings that the major route of disposal of acetate *in vivo* is by oxidation in the peripheral tissues (Wick & Drury, 1952; Lundquist, Tygstrup, Winkler, Møllergaard & Munck-Petersen, 1962). Although acetate is not a major normal substrate for non-ruminants, an active acetate thiokinase has been shown to be present in pig heart muscle (Beinert *et al.* 1953), and the oxidation of acetate has been shown to proceed rapidly by way of the citric acid cycle in guinea-pig-heart mitochondria, with the accumulation of citrate at high acetate concentrations (Maley & Plaut, 1953).

There was appreciable incorporation of ^{14}C from $[2-^{14}\text{C}]$ acetate into citric acid and glutamic acid in the heart after a 20 min. perfusion period. A similar preferential incorporation of $[2-^{14}\text{C}]$ acetate into glutamic acid, glutamine and citric acid relative to other amino acids and citric acid-cycle intermediates has been reported by Huggins & Smith (1962) with rat diaphragm incubated in Krebs-Ringer bicarbonate. Reversible transamination between glutamic acid and aspartic acid in perfused rat heart has been reported by Hicks & Kerly (1960), and Borst (1962) demonstrated high glutamate-oxaloacetate-transaminase activity but no glutamate-dehydrogenase activity in rat-heart mitochondria. When hearts were perfused with substrate-free medium, the high glutamate concentration initially present in the heart decreased slightly, whereas the aspartate disappeared completely (Hicks & Kerly, 1960). It would appear that glutamate and aspartate are in equilibrium with their respective oxo acids, and the available evidence suggests that the rapid incorporation of ^{14}C from $[2-^{14}\text{C}]$ acetate into glutamate may be due to transamination between aspartate and α -oxoglutarate, possibly caused by an increased concentration of α -oxoglutarate in the presence of

acetate. Alternatively, ^{14}C -exchange reactions between glutamate and α -oxoglutarate, not involving glutamate-oxaloacetate transaminase, may explain the observed ^{14}C -labelling, as discussed by Haslam & Krebs (1963). The incorporation of ^{14}C into the tissue pools of amino acids and citric acid-cycle intermediates probably accounts for the lag in $^{14}\text{CO}_2$ production with $[2\text{-}^{14}\text{C}]\text{acetate}$ as substrate in the present experiments.

Insulin stimulated the uptake of acetate by 20 % and decreased the intracellular acetate concentration after a 30 min. perfusion period. This effect suggests that insulin is not changing the membrane permeability to acetate, but either directly or indirectly is affecting an intracellular process, such as the rate of conversion of acetate into acetyl-CoA. An increased availability of ATP, which is necessary for the acetate-thiokinase reaction, could also explain this effect. It remains to be evaluated whether the stimulation of acetate uptake by insulin reflects a more fundamental action, or whether it has no physiological significance.

Pyruvate (10 mM) was somewhat more readily oxidized than acetate, and accounted for about 90 % of the fuel of respiration, in agreement with results reported by Clarke (1957) with a blood-perfused rat-heart preparation. Although intracellular pyruvate concentrations were not measured, the rapid rate of pyruvate utilization suggests that pyruvate, like acetate, is not subject to cellular permeability barriers. In the presence of pyruvate (unlike acetate) appreciable quantities of lactate appeared in the medium. Glycogen concentrations in the heart remained constant during perfusion. Hence glycogen breakdown did not contribute to medium lactate, which was formed from exogenous pyruvate, and accounted for about 30 % of the pyruvate uptake. Since lactate dehydrogenase appears to be extramitochondrial in its distribution (de Duve, Wattiaux & Baudhuin, 1962), the continuous production of lactate from pyruvate implies the operation of a mechanism for the regeneration of cytoplasmic NADH not involving glycolysis. Mitochondria are not sufficiently permeable to NADH to allow a direct transfer of NADH from mitochondria to cytoplasm, but a more reduced state of the mitochondrial NAD system could be extended to the cytoplasmic NAD^+ by means of a substrate-hydrogen cycle (Boxer & Devlin, 1961; Borst, 1963). The nature of the substrate couple has not been firmly established for mammalian tissues, but the malate-aspartate cycle proposed by Borst (1961), which involves a double transamination between aspartate and glutamate, is of particular interest.

Pyruvate was also a major fuel at perfusate concentrations of 0.2–1.0 mM, but below 0.1 mM, which is the normal plasma pyruvate concentration, pyruvate contributed only 5–10 % to the total

energy requirement of the heart, in agreement with the findings *in vivo* by Goodale & Hackel (1953) in the dog, and by Goodale *et al.* (1959) in man.

Effects of starvation. Starvation of rats for 24 hr. had no effect on the acetate uptake of the perfused rat heart. This finding is in accordance with the lack of an effect of starvation on acetoacetate metabolism (Williamson & Krebs, 1961) and on palmitate metabolism (Opie, Evans & Shipp, 1963) in perfused rat hearts. Rat diaphragm and skeletal muscle apparently behave somewhat differently in that the oxidation of long-chain but not short-chain fatty acids is increased by starvation or diabetes (Fritz, 1961).

Starvation of rats for 24 or 48 hr. decreased the pyruvate uptake of perfused rat hearts relative to the fed controls. The proportion of the pyruvate taken up converted into lactate was increased, and the contribution of pyruvate to the fuel of respiration was decreased from 90 to 71 %. Similar observations have been reported by Garland, Newsholme & Randle (1962), who showed that hearts from diabetic rats likewise have impaired utilization of pyruvate. When pyruvate is derived from glucose or glycogen, the decreased rate of pyruvate oxidation with hearts from starved rats is reflected by an increased rate of lactate and pyruvate formation and a decreased lactate:pyruvate concentration ratio (Williamson, 1962*a*; Garland *et al.* 1962; Williamson, Walker & Renold, 1963; Opie *et al.* 1963). Blood pyruvate and lactate concentrations have been found to be elevated in diabetic subjects, and the blood pyruvate concentrations fall more slowly in diabetic than in normal subjects after a glucose and insulin or pyruvate tolerance load, suggesting a similar impairment of pyruvate oxidation *in vivo* (Fry & Butterfield, 1962; Moorhouse, 1959).

Insulin increased the uptake of pyruvate by hearts from starved rats to values that were the same as those obtained with fed rats. This effect is similar to that observed by Pearson *et al.* (1949) and Vilee & Hastings (1949), who used a diaphragm preparation from normal and diabetic rats. Lactate uptake by the perfused rat heart, which was decreased 50 % by starvation, was not affected by insulin, however (Williamson, 1962*a*). The mechanism responsible for the basic defect of pyruvate utilization in muscle tissues from starved and diabetic animals, and its apparent reversal by insulin, remain to be clarified. The defect may be due primarily either to hormonal or metabolic factors. Pituitary and adrenal-cortical hormones have been shown by Park *et al.* (1961) to be involved in the decreased rate of glucose and fructose 6-phosphate phosphorylation exhibited by hearts from diabetic rats perfused with glucose as the sole exogenous nutrient, and the pyruvate-oxidative-

decarboxylase system may also be subject to inhibition by these humoral factors in the presence of low blood concentrations of insulin, or simply be inhibited by insulin deficiency.

Alternatively, inhibition of pyruvate metabolism may be secondary to enhanced respiration of triglycerides, ketone bodies and fatty acids (Garland *et al.* 1962). The administration of growth hormone to intact dogs has been shown by Weil, Altszuler & Kessler (1961) to increase the concentration of pyruvate in the blood, and to produce a delayed fall of the pyruvate concentration after glucose and pyruvate infusions. However, as these authors point out, a possible direct effect of growth hormone on an enzyme process concerned with pyruvate metabolism could not be distinguished from secondary effects due to its lipolytic activity, resulting in more fatty acids as an alternative respiratory fuel to pyruvate. Similar effects on blood pyruvate concentrations and pyruvate tolerance in man with adrenal corticosteroids have been reported by Fajans (1961). Insulin may either relieve the postulated inhibition of the pyruvate-decarboxylase reaction directly, or as a secondary effect to an anti-lipolytic action, in a manner analogous to that observed in adipose tissue (Mahler, Tarrant, Stafford & Ashmore, 1963; Jungas & Ball, 1963; Perry & Bowen, 1962).

Competition for metabolism between glucose, acetate and pyruvate. The metabolism of glucose and pyruvate by isolated hearts from normal fed rats may be made to resemble that of hearts from starved or diabetic rats by the addition to the perfusate of substances that are not subject to permeability restrictions, and are readily metabolized to acetyl-CoA. In the absence of insulin, glucose uptake is either unchanged or slightly decreased by the addition of acetoacetate, fatty acids, acetate, lactate or pyruvate to the perfusate, while lactate formation and glycogen synthesis are increased, and glucose oxidation is almost abolished (Williamson & Krebs, 1961; Williamson, 1962*a*, and unpublished work; Shipp *et al.* 1961). When insulin is added to the perfusate, glucose uptake, although stimulated above the non-insulin controls, is inhibited 50–60 % by the presence of these compounds. In the present studies, acetate or pyruvate added to the perfusate in addition to glucose and insulin increased glycogen synthesis above that observed with glucose and insulin alone, and most of the glucose removed from the medium was accounted for as lactate or glycogen. Under these circumstances, the utilization of pyruvate or acetate was decreased by only 10–15 %. Acetate or pyruvate in the absence of glucose also prevented the glycogenolysis normally observed in hearts from starved rats perfused with glucose alone. On the basis of changes in the concentrations of hexose mono- and di-phosphates in hearts per-

fused with glucose, and ketone bodies, fatty acids or pyruvate, it has been proposed that the decreased rate of glucose uptake observed in the two situations is caused by a common mechanism, namely diminished activity of phosphofructokinase (Newsholme, Randle & Manchester, 1962; Randle & Morgan, 1962). However, there are noticeable differences in these metabolic states, particularly with regard to glycogen metabolism and the lactate: pyruvate concentration ratio. Although hearts taken from starved and diabetic rats contain high concentrations of glycogen compared with hearts from fed rats, the rate of glycogenolysis by hearts from starved rats has been shown to be much increased on subsequent perfusion with glucose as the only exogenous substrate (Williamson, 1962*a*). Thus, though elevated glucose 6-phosphate concentrations in hearts from starved rats (Newsholme & Randle, 1961) are associated with glycogenolysis, elevated glucose 6-phosphate concentrations in hearts from fed rats perfused with acetate, pyruvate, fatty acids or ketone bodies (in the presence or absence of insulin) are associated with glycogen synthesis (Newsholme *et al.* 1962; J. R. Williamson, unpublished work).

The uptake of pyruvate (10 mM) by hearts from fed rats was inhibited 20 % by an equimolar concentration of acetate, and acetate uptake was decreased by 53 %. In the experiments of Olson (1962) the pyruvate uptake of perfused hearts from fed rats was little inhibited by 0.27 mM-fatty acids bound to 2 % albumin, but the oxidation of fatty acids was decreased by 90 %, and the incorporation of exogenous fatty acids into neutral lipid was increased 5–6-fold. Higher concentrations (0.75 mM) of albumin-bound fatty acids, however, have been shown to inhibit pyruvate utilization (Garland *et al.* 1962; Evans, Opie & Renold, 1963). Competition between pyruvate and acetate or fatty acids could either be at the level of cellular penetration or at the intramitochondrial site of entry into the citric acid cycle. The former possibility is considered unlikely: first because the proportion of the pyruvate uptake converted into lactate is increased in the presence of acetate or fatty acids (Garland *et al.* 1962; Evans *et al.* 1963), and secondly because pyruvate causes an immediate reduction of mitochondrial nicotinamide nucleotide as a result of the formation of acetyl-CoA from pyruvate (Williamson & Chance, 1964), showing that penetration into the cell is rapid. Competition for entry into the citric acid cycle may be explained on the basis of competition between the metabolites for the available CoA, and it would appear that the rate of oxidation of a particular metabolite is largely determined by the rate at which it can be converted into acetyl-CoA. This will depend on the relative concentrations of the precursors and the rate of their disposal by

alternative pathways, e.g. to lactate, glycogen or triglyceride.

The concept that the defective metabolism of carbohydrate by muscle tissues of starved and diabetic animals is secondary to enhanced respirations of fatty acids and ketone bodies has been emphasized by Randle, Garland, Hales & Newsholme (1963). In isolated tissues perfused or incubated in the absence of exogenous free fatty acids, the only source of fatty acids is from the breakdown of endogenous fat. Catabolism of endogenous fat is increased in hearts from starved or diabetic rats perfused with glucose medium compared with fed controls as indicated by an increased rate of glycerol release into the perfusate, and increased tissue concentrations of fatty acids and fatty acyl-CoA derivatives (Randle *et al.* 1963; Garland & Randle, 1963; Garland, Randle & Newsholme, 1963). Suppression of glucose oxidation in the starved state would be expected to increase endogenous fat oxidation by 25 %, since total respiration is not affected by starvation (Williamson, 1962*a*). Exogenous free fatty acids are relatively poorly oxidized compared with pyruvate (Evans *et al.* 1963), and hence it appears unlikely that enhanced breakdown of endogenous fat in muscle tissues from starved or diabetic rats would be sufficient to account for the observed decrease of pyruvate uptake. Further, Bjorntorp & Furman (1962) observed no increased activity of tissue lipase in heart homogenates from starved rats. If increased lipolysis were the cause of the decreased pyruvate uptake in starved and diabetic muscle, the increased intracellular concentration of free fatty acids would also be expected to inhibit acetate, fatty acid or acetoacetate uptake, but no such effect is observed. These observations suggest that increased lipolysis may in fact be secondary to a primary defect of pyruvate oxidation. Clearly, the interactions between carbohydrate and fat or fatty acid oxidation even in an isolated muscle preparation not subject to hormonal influence are complex, and it would appear that decreased carbohydrate utilization in the starved or diabetic states is due in part to increased availability of alternative fuels, and in part to a basic defect in the ability of the tissue to convert carbohydrate into acetyl-CoA.

SUMMARY

1. Acetate oxidation by perfused hearts from fed rats accounted for about 75 % of the respiratory fuel over the concentration range 1–10 mM. Pyruvate was also readily utilized and was the major respiratory fuel when present in the perfusate at concentrations above 0.2 mM. At high concentrations (2–10 mM) about 30 % of the pyruvate removed from the medium was converted into lactate.

2. The uptake of pyruvate by hearts from rats starved for 24 or 48 hr. was significantly less than that from fed rats, and the proportion of pyruvate converted into lactate was increased. The estimated contribution of pyruvate to the total fuel of respiration was decreased by starvation from 90 to 70 %. Addition of insulin to hearts from starved rats restored to normal the rate of pyruvate uptake.

3. Acetate uptake was not affected by starvation, but was increased 20 % by insulin.

4. The pyruvate uptake by hearts from fed rats was decreased 20 % in the presence of acetate. Glucose uptake in the presence of insulin was decreased 40–45 % by acetate or pyruvate, and the conversion of glucose into lactate and glycogen was increased. Under these conditions acetate or pyruvate utilization was decreased by only 10–15 %.

5. These studies indicate that increased availability of alternative respiratory fuels inhibit carbohydrate utilization in muscle by competition for the available coenzyme A. The possible relation between this effect and the decreased utilization of carbohydrate by hearts from starved and diabetic animals is discussed.

This work was aided by grants-in-aid from the Rockefeller Foundation, the U.S. Public Health Service in England; in America this work was supported by grants-in-aid from the U.S. Public Health Service (5T1 AM-5077-08 and AM-05493) and the Life Insurance Medical Research Fund Grant G 61-9. The author is indebted to Professor Sir Hans Krebs, F.R.S., for his helpful criticisms.

REFERENCES

- Beinert, H., Green, D. E., Hele, P., Hift, H., Korff, R. W. von & Ramakrishnan, C. V. (1953). *J. biol. Chem.* **203**, 35.
- Bing, R. J. (1954–55). *Harvey Lect.* **50**, 27.
- Bjorntorp, P. & Furman, R. H. (1962). *Amer. J. Physiol.* **203**, 323.
- Borst, P. (1961). *Proc. 5th int. Congr. Biochem., Moscow*, vol. 2, p. 233. Oxford: Pergamon Press Ltd.
- Borst, P. (1962). *Biochim. biophys. Acta*, **57**, 256.
- Borst, P. (1963). *Funktionelle und morphologische Organisation der Zelle*, p. 137. Berlin: Springer-Verlag.
- Boxer, G. E. & Devlin, T. M. (1961). *Science*, **134**, 1494.
- Clarke, E. W. (1957). *J. Physiol.* **136**, 380.
- de Duve, C., Wattiaux, R. & Baudhuin, P. (1962). *Advanc. Enzymol.* **24**, 291.
- Evans, J. R., Opie, L. H. & Renold, A. E. (1963). *Amer. J. Physiol.* **205**, 971.
- Fajans, S. S. (1961). *Recent Progr. Hormone Res.* **17**, 534.
- Fisher, R. B. & Lindsay, D. B. (1956). *J. Physiol.* **131**, 526.
- Fisher, R. B. & Williamson, J. R. (1961). *J. Physiol.* **158**, 102.
- Fritz, I. B. (1961). *Physiol. Rev.* **41**, 53.
- Fry, I. K. & Butterfield, W. J. H. (1962). *Lancet*, ii, 66.
- Garland, P. B., Newsholme, E. A. & Randle, P. J. (1962). *Nature, Lond.*, **195**, 381.
- Garland, P. B. & Randle, P. J. (1963). *Nature, Lond.*, **199**, 381.

- Garland, P. B., Randle, P. J. & Newsholme, E. A. (1963). *Nature, Lond.*, **200**, 169.
- Goodale, W. T. & Hackel, D. B. (1953). *Circulation Res.* **1**, 509.
- Goodale, W. T., Olson, R. E. & Hackel, D. B. (1959). *Amer. J. Med.* **27**, 212.
- Haslam, R. J. & Krebs, H. A. (1963). *Biochem. J.* **88**, 566.
- Hicks, R. M. & Kerly, M. (1960). *J. Physiol.* **150**, 621.
- Holzer, H. & Holldorf, A. (1957). *Biochem. Z.* **329**, 229.
- Huggins, A. K. & Smith, M. J. M. (1962). *Biochem. J.* **85**, 394.
- Hülsmann, W. C. (1960). *Biochim. biophys. Acta*, **45**, 623.
- Jungas, R. L. & Ball, E. G. (1963). *Biochemistry*, **2**, 383.
- Kipnis, D. M., Helmreich, E. & Cori, C. F. (1959). *J. biol. Chem.* **234**, 165.
- Krahl, M. E. & Cori, C. F. (1947). *J. biol. Chem.* **170**, 607.
- Kreisberg, R. A. & Williamson, J. R. (1964). *Amer. J. Physiol.* (in the Press).
- Large, P. J., Peel, D. & Quayle, J. R. (1961). *Biochem. J.* **81**, 470.
- Lundquist, F. (1962). *Nature, Lond.*, **193**, 579.
- Lundquist, F., Tygstrup, N., Winkler, K., Mellemegaard, K. & Munck-Peterson, S. (1962). *J. clin. Invest.* **41**, 955.
- Mahler, R., Tarrant, M. E., Stafford, W. S. & Ashmore, J. (1963). *Diabetes*, **12**, 359.
- Maley, G. F. & Plaut, G. W. E. (1953). *J. biol. Chem.* **205**, 297.
- Moorhouse, J. A. (1959). *J. Lab. clin. Med.* **54**, 927.
- Morgan, H. E., Cadenas, E., Regen, D. M. & Park, C. R. (1961). *J. biol. Chem.* **236**, 262.
- Morgan, H. E., Henderson, M. J., Regen, D. M. & Park, C. R. (1961). *J. biol. Chem.* **236**, 253.
- Newsholme, E. A. & Randle, P. J. (1961). *Biochem. J.* **80**, 655.
- Newsholme, E. A., Randle, P. J. & Manchester, K. L. (1962). *Nature, Lond.*, **193**, 270.
- Olson, R. E. (1962). *Nature, Lond.*, **195**, 597.
- Olson, R. E. & Piatnek, D. A. (1959). *Ann. N.Y. Acad. Sci.* **72**, 466.
- Opie, L. H., Evans, J. R. & Shipp, J. C. (1963). *Amer. J. Physiol.* **205**, 1203.
- Park, C. R., Morgan, H. E., Henderson, M. J., Regen, D. M., Cadenas, E. & Post, R. L. (1961). *Recent Progr. Hormone Res.* **17**, 493.
- Pearson, O. H., Hsieh, C. K., Dutoit, C. H. & Hastings, A. B. (1949). *Amer. J. Physiol.* **158**, 261.
- Perry, W. F. & Bowen, H. F. (1962). *Canad. J. Biochem. Physiol.* **40**, 749.
- Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963). *Lancet*, **i**, 785.
- Randle, P. J. & Morgan, H. E. (1962). *Vitam. & Horm.* **20**, 199.
- Sakami, W. (1955). *Handbook of Isotope Tracer Methods*, p. 1. Cleveland, Ohio: Department of Biochemistry, Western Reserve University.
- Serlin, I. & Cotzias, G. C. (1952). *J. biol. Chem.* **215**, 263.
- Shipp, J. C., Opie, L. H. & Challoner, D. (1961). *Nature, Lond.*, **189**, 1018.
- Taylor, T. G. (1953). *Biochem. J.* **54**, 48.
- Villee, C. A. & Hastings, A. B. (1949). *J. biol. Chem.* **181**, 131.
- Weil, R., Altszuler, N. & Kessler, J. (1961). *Amer. J. Physiol.* **201**, 251.
- Wick, A. N. & Drury, D. R. (1952). *J. biol. Chem.* **199**, 127.
- Williamson, J. R. (1961). *Biochem. J.* **81**, 16 p.
- Williamson, J. R. (1962a). *Biochem. J.* **83**, 377.
- Williamson, J. R. (1962b). *Fed. Proc.* **21**, 320.
- Williamson, J. R. & Chance, B. (1964). *Abstr. 6th int. Congr. Biochem., New York* (in the Press).
- Williamson, J. R. & Krebs, H. A. (1961). *Biochem. J.* **80**, 540.
- Williamson, J. R., Walker, R. A. & Renold, A. E. (1963). *Metabolism*, **12**, 1141.
- Winegrad, A. I. & Renold, A. E. (1958). *J. biol. Chem.* **233**, 267.

Biochem. J. (1964), **93**, 106

The Interaction between Polysaccharides and other Macromolecules

9. THE EXCLUSION OF MOLECULES FROM HYALURONIC ACID GELS AND SOLUTIONS*

By TORVARD C. LAURENT

Department of Medical Chemistry, University of Uppsala, Sweden

(Received 2 March 1964)

The extent to which hyaluronic acid can exclude other macromolecules from its molecular domain has long been a matter of controversy. Johnston (1955) calculated an impenetrable volume for albumin of 10^3 ml./g. of polysaccharide from sedimentation experiments with albumin and hyaluronic acid, but his results may be due to effects

other than an exclusion (Laurent & Pietruszkiewicz, 1961). Blumberg & Ogston (1956) and Ogston & Phelps (1961) studied the sedimentation of hyaluronic acid in the presence of various concentrations of serum albumin and β_A -lactoglobulin. Their experiments indicated that only 12 ml. of solvent/g. of polysaccharide could be considered inaccessible to the proteins. However, simultaneous experiments with equilibrium dialysis

* Part 8: Laurent & Killander (1964).