BRITISH MEDICAL JOURNAL

LONDON SATURDAY OCTOBER 17 1959

RECENT STUDIES ON CARBOHYDRATE METABOLISM*

BY

E. B. CHAIN, M.A., Ph.D., F.R.S.

Head of the Departments of Biochemistry and Chemical Microbiology, Istituto Superiore di Sanità, Rome

I wish to express my sincere thanks to the President and the Council of the Harveian Society of London for the honour they have done me in inviting me to give this year's Harveian Lecture.

While I deeply appreciated having the offer of this great privilege I had some considerable doubts in my mind of my qualifications for the task. Nearly all the previous Harveian lectures have been concerned with purely medical subjects, usually reviewing advances in some important field of clinical medicine, and, almost without exception, they were presented by famous physicians or surgeons. As you know, I am a biochemist by profession, without medical training, and, moreover, my personal biochemical interests have never been primarily concerned with problems of clinical medicine.

However, I finally found the courage to accept the invitation of the Harveian Society, mainly on the strength of the reflection that, after all, some major advances in modern medicine—for instance, much of the knowledge which led to the therapeutic use of vitamins, antibiotics, and some hormones—were the results of pure academic biochemical investigations which did not directly originate from the daily problems of the hospital ward, though I am very conscious of the fact that the primary impulse to these studies has usually been a biological or clinical observation, and that for some aspects of medical research the closest contact between the clinician and the biochemist is not only useful but essential for progress.

I have always been particularly interested in the elucidation of the biochemical mode of action of substances which display some characteristic biological action. This led me to the study of the mode of action of the neurotoxic principle contained in some snake venoms, and in collaboration with various colleagues, particularly H. W. (now Sir Howard) Florey and Dr. E. S. Duthie,† to the study of the biochemical mechanism of some lytic and permeability phenomena, and, as an expansion of those investigations, to the study of antibacterial substances produced by micro-organisms, now known as antibiotics, which resulted in the discovery of the curative power of Fleming's penicillin.

About 10 years ago I and my colleagues at the biochemistry department of the Istituto Superiore di Sanità (the Institute of Public Health of the Italian State) began an investigation into the mode of action of insulin, as part of a more general research programme on the mode of action of hormones. We felt that a

deeper understanding of the mode of action of these substances, which, in minute amounts and in a specific manner, regulate the rates of metabolic reactions, was essential for further progress in the understanding and, eventually, the treatment of metabolic disorders, and therefore represented one of the most central and important fundamental problems of modern biochemistry.

In-vitro Effect of Insulin on Glycogen Synthesis in Rat Diaphragm

We chose insulin for the start of this study because in 1949, when this work was initiated, at least one clear-cut *in-vitro* effect of insulin on an isolated tissue had been conclusively demonstrated: Gemmill (1940, 1941) had shown that in the isolated rat diaphragm insulin stimulates glucose uptake and glycogen synthesis. We thought that so much progress in the understanding of the reactions of intermediate carbohydrate metabolism had been made and so many new powerful analytical tools for its study had become available that it should either be possible to fit insulin into the existing reaction schemes, or else some new important reactions which had hitherto escaped attention would come to light.

The effect of insulin on glycogen synthesis and glucose uptake in the isolated rat diaphragm discovered by Gemmill was confirmed by a number of authors, including Vilee and Hastings (1949), who were the first to use radioactive glucose for these studies. From the figures of these experiments, given in Table I, it is evident that only a small part of the glucose metabolized was recovered as glycogen and CO₂; the greater part was unaccounted for, and this "unaccounted for" fraction was greater in the presence of insulin than in its absence.

TABLE I.—*In-vitro* Utilization of ¹⁴C-labelled Glucose by Rat-diaphragm Muscle (after Vilee and Hastings, 1949)

	Normal		Diabetic	
	Without Insulin	With Insulin	Without Insulin	With Insulin
No. of animals	14	14	7	7
Total glucose disappearing from medium* (mg./g./hr.)	1.53 ±0.046	2.87 ±0.047	0.78 ±0.035	2.17 ±0.10
Glucose metabolized to CO ₂ * (mg./g./hr.)	0.11 ±0.012	0.22 ±0.026	0.08 ±0.019	0.11 ±0.030
Glycogen synthesized* (mg./g./hr.)	0.17 ±0.062	0.62 ±0.13	0.14 ±0.045	0.61 ±0.19
Glucose unaccounted for (mg./g./hr.)	1.25	2.03	0.56	1.45
Utilized glucose recovered as CO ₂	7.2	7.7	10.3	5.1
Utilized glucose converted to glycogen (%)	11.1	21.6	18.0	28.1
CO ₂ in centre well derived from glucose in medium (%)	10.4	16.3	8.1	9.2

* The values given are means ± standard error.

*Harveian Lecture delivered to the Harveian Society of London on March 18, 1959.

†Dr. Duthie died suddenly in May, 1959. His untimely death has caused profound grief to myself and his many friends and is a great loss to science.

Quantitative Radiochromatographic Techniques

My colleagues and I set ourselves the task of getting a quantitative balance-sheet of glucose metabolism in the isolated diaphragm of the rat in the absence and presence of insulin (Beloff-Chain, Catanzaro, Chain, Masi, Pocchiari, and Rossi, 1955a). After incubation of the muscle for various periods with generally labelled ^{14}C -glucose on other labelled substrates of carbohydrate metabolism, the radioactive metabolites in the incubation medium and the tissue were separated by suitable paper chromatographic methods and quantitatively determined. In addition, the CO_2 formed was collected and its radioactivity measured. The quantitative evaluation of the chromatograms was first done by hand, using the method of Fink, Dent, and Fink (1947), by passing paper strips under a Geiger counter and measuring the radioactivity per centimetre. This method proved too slow and tedious for monodimensional chromatograms, and impracticable for

typewriter carriage between two end-window Geiger counters attached to the back of the typewriter, thus exposing a unit square area of the radiochromatogram for counting for a period depending on the intensity of the radiations. If the radiation intensity of the unit area of the paper chromatogram exposed to the Geiger counters is below a certain arbitrarily chosen threshold value, this is considered background radiation and the typewriter carriage is held stationary for 45 seconds; if this threshold value is exceeded it is considered to be due to radioactive material and the typewriter carriage is maintained stationary for five times this period.

An electronic discrimination circuit transmits the number of counts, collected in the Geiger counters and counted in electronic counting-tubes, to the corresponding number keys of the typewriter, and at the end of the counting period the number is printed on the blank sheet in the typewriter carriage in the position corresponding to the position of the radioactive spot on the radiochromatogram; the typewriter carriage then moves on to the next position, and when the whole line is explored returns to its original position, simultaneously turning the roll so that the blank paper sheet and with it the paper chromatogram are moved toward the next line.

The time necessary for the complete evaluation of a bidimensional radiochromatogram depends on the radioactivity; under the experimental conditions used by us the evaluation of a bidimensional radiochromatogram of a tissue extract incubated with a ^{14}C - or ^{32}P -labelled metabolite takes about 16 hours. Six scanning machines are in continuous use in our laboratory; they function day and night, sometimes for weeks without interruption, and require very little maintenance.

A scanning device for monodimensional radiochromatograms using the same "read-out" method has also been devised (Fig. 2).

In this way "number maps" are obtained which give the quantitative distribution of the radioactive metabolites separated on the bidimensional radiochromatograms (Fig. 3). With this technique, each tissue after incubation with radioactive substrates was found to have a characteristic qualitative and quantitative metabolic pattern reflecting its biochemical functional state. Characteristic changes of this pattern are observed in pathological conditions and under the influence of hormones and drugs; on the basis of these changes deductions can be made of the nature of the

FIG. 1.—Automatic scanning device for bidimensional radiochromatograms (Frank *et al.*, 1959).

bidimensional chromatograms. Consequently it became necessary to develop automatic scanning methods. This was done in collaboration with Dr. M. Frank, of the electronics department of the institute. The older methods, described in previous publications from this department (Chain, Frank, Pocchiari, Rossi, Ugolini, and Ugolini, 1956), are now superseded by the latest model (Fig. 1) (Frank, Chain, Pocchiari, and Rossi, 1959).

The scanning apparatus consists of an electric typewriter on the number of keys of which a series of solenoids is fixed, actuating them when energized. The radiochromatogram is fastened by Scotch-tape to a sheet of blank paper inserted into the carriage of the typewriter and is drawn by the movements of the

FIG. 2.—Automatic scanning device for monodimensional radiochromatograms.

FIG. 3.—Radioautograph and the corresponding number map of a bidimensional radiochromatogram.

"biochemical lesion" (Peters, 1936) on the point of attack of the pharmacologically active substances.

Action of Insulin on Glucose Metabolism in the Isolated Diaphragm Muscle of the Rat

Radioautograms of monodimensional radiochromatograms of the incubation medium and of an aqueous-alcoholic extract of exhaustively washed rat-diaphragm muscle after incubation for 90 minutes with uniformly labelled glucose are shown in Fig. 4. The main metabolite in the incubation medium was lactate; in addition triosephosphates were found. The tissue extract also contained lactate, though much less than the incubation medium; in addition, glucose, glycogen, and a number of oligosaccharides were found. The latter are better separated by bidimensional chromatography and were identified as maltose, maltotriose, and maltotetraose (Fig. 5). The insoluble residue of the muscle after extraction with aqueous alcohol was also radioactive; nearly all the radioactivity was accounted for as glycogen. Thus, the "unaccounted for" glucose in the table of Villee and Hastings (1949) was recovered as lactate, intracellular glucose, oligosaccharides, and insoluble glycogen.

FIG. 4.—Radiochromatogram of aqueous-alcoholic extract of diaphragm muscle of the rat and media before and after incubation with ^{14}C -labelled glucose. A_1 = Medium before incubation. A_2 = Medium after incubation.

This insulin effect takes place very rapidly, being already noticeable after only three minutes' incubation (Figs. 6 and 7).

Fructose (Beloff-Chain, Catanzaro, Chain, Masi, and Pocchiari, 1955b) forms the same metabolites in rat-diaphragm muscle as does glucose, but only half as much total fructose as glucose is metabolized, and the

TABLE II.—Influence of Insulin on Glucose Metabolism in Rat-diaphragm Muscle*

	No Insulin	With Insulin
Aqueous-alcoholic extract:		
Zone ps	10.4 $\pm$ 1.7	23.7 $\pm$ 2.8
" m	3.0 $\pm$ 0.3	5.6 $\pm$ 0.7
" g	18.0 $\pm$ 1.9	21.6 $\pm$ 2.0
Glycogen of insoluble residue ..	14.9 $\pm$ 4.3	25.4 $\pm$ 6.7
Zone 1 (sum of lactate in extract and medium)	24.8 $\pm$ 3.4	28.4 $\pm$ 4.9
CO_2	13.8 $\pm$ 1.9	13.6 $\pm$ 2.0

* Results expressed as μg . of glucose converted per 50 mg. of diaphragm muscle (wet weight) incubated in 0.6 ml. of phosphate buffered medium at pH 6.8 at 37°C . Glucose concentration 0.1%. Mean values of 8 experiments given $\pm$ S.E.M. (after Beloff-Chain *et al.*, 1955a).

FIG. 5.—Radiochromatogram of aqueous-alcoholic extract of diaphragm muscle of the rat incubated with ^{14}C -labelled glucose. (Two-dimensional chromatogram: $\leftarrow$ butanol: ethanol: NH_3 : water; $\downarrow$ picric acid: tertiary butyl-alcohol: water.) (Beloff-Chain *et al.*, 1955a.)

formation of glycogen and oligosaccharides is reduced to about one-seventh. CO_2 production and production of lactic acid from fructose are about the same as they are from glucose.

As in the case of glucose, insulin has no effect on the catabolic reactions of fructose, but a marked effect on the anabolic reactions leading to the formation of glycogen and oligosaccharides (Table III).

TABLE III.—Influence of Insulin on Fructose Metabolism in Rat-diaphragm Muscle*

	No Insulin	With Insulin
Aqueous alcoholic extract:		
Zone ps	1.4 $\pm$ 0.4	5.0 $\pm$ 0.9
" m	0.7 $\pm$ 0.09	1.9 $\pm$ 0.2
" f	27.4 $\pm$ 1.4	29.0 $\pm$ 3.2
Glycogen of insoluble residue ..	1.6 $\pm$ 0.3	3.3 $\pm$ 0.7
Zone 1 (sum of lactate in extract and medium)	31.4 $\pm$ 1.0	35.2 $\pm$ 0.4
CO_2	13.7 $\pm$ 1.1	13.6 $\pm$ 0.8

* Results expressed as μg . of fructose converted per 50 mg. of diaphragm muscle (wet weight) incubated in 0.6 ml. of phosphate buffered medium at pH 6.8 at 37°C . Fructose concentration 0.1%. Mean values of 4 experiments given $\pm$ S.E.M. (After Beloff-Chain *et al.*, 1955b.)

Action of Insulin on Glucose Metabolism in the Isolated Brown Adipose Tissue of the Rat

Active synthesis of glycogen and fat has been shown by Tuerkischer and Wertheimer (1942) to occur *in vivo* in the brown adipose tissue of the rat, and Wertheimer (1945) demonstrated that after injection of insulin the synthesis of glycogen in this tissue was greatly

stimulated. We followed the fate of uniformly labelled glucose in slices of brown adipose tissue of the rat with their quantitative radiochromatographic technique in the presence and absence of insulin (Beloff-Chain, Catanzaro, Chain, Masi, and Pocchiari, 1956a). As shown in Table

FIG. 6. — Radiochromatogram of aqueous-alcoholic extract of diaphragm muscle of the rat incubated for varying periods with uniformly labelled ^{14}C -glucose (Beloff-Chain *et al.*, 1955a).

shown to stimulate two synthetic reactions—the synthesis of glycogen and oligosaccharides, and fat synthesis, the most endergonic of all metabolic syntheses.

TABLE IV.—Influence of Insulin on Glucose Metabolism in Slices of Brown Adipose Tissue of the Rat*

	No Insulin	With Insulin
Aqueous alcoholic extract:		
Zone ps + m	1.0 $\pm$ 0.08	2.7 $\pm$ 0.4
" g	12.9 $\pm$ 1.2	11.2 $\pm$ 1.3
" al	7.7 $\pm$ 0.9	9.8 $\pm$ 1.5
Insoluble residue (fat)	8.1 $\pm$ 1.3	15.2 $\pm$ 1.6
Zone I (sum of lactate in extract and medium)	48.6 $\pm$ 6.3	69.3 $\pm$ 4.8
CO_2	52.0 $\pm$ 7.1	61.0 $\pm$ 6.7

* Results expressed as $\mu\text{g.}$ of glucose converted per 50 mg. of diaphragm muscle (wet weight) incubated in 0.6 ml. of phosphate buffered medium at pH 6.8 at 37° C. Glucose concentration 0.1%. Mean values of 11 experiments given $\pm$ S.E.M. (After Beloff-Chain *et al.*, 1956a.)

Current Theories of Insulin Action

Two theories have been advanced for the mode of action of insulin—the "permeability" theory and the "hexokinase" theory. According to the first, widely accepted, theory, insulin acts by increasing the permeability of the cell to glucose; according to the second it stimulates the hexokinase reaction—that is, the formation of glucose-6-phosphate from adenosine triphosphate (ATP). In our view neither of these theories can be reconciled with the experimental facts.

Experiments Designed to Test the Permeability Theory

If insulin acted by increasing the permeability of the cell to glucose—that is, by increasing the glucose concentration inside the cell—it should be possible to

simulate the insulin effect by increasing the concentration of glucose in the incubation medium, provided it could be shown that the intracellular concentration of glucose increased to about the same degree as the extracellular concentration. Fig. 8 gives the results of measuring the concentration of uniformly labelled ^{14}C -glucose in the rat diaphragm after incubation with increasing amounts of uniformly labelled ^{14}C -glucose and subsequent careful washing. An aqueous-alcoholic extract of the muscle was prepared, the glucose separated chromatographically on paper and determined by measuring its radioactivity. It will be seen that the "intracellular" glucose concentration increases linearly with the external glucose concentration in the incubation medium.

In Table V the effect of increasing the glucose concentration in the incubation medium on the different glucose metabolites formed by the rat diaphragm is shown. Unlike insulin, an increase of the external glucose concentration brings about a marked increase of CO_2 and lactic acid production. Oligosaccharides and glycogen also increase when the concentration of

glucose in the incubation medium is raised from 0.1 to 0.5% (actually the limiting glucose concentration at which a stimulating effect on oligosaccharide and polysaccharide synthesis is noted is about 0.3%), and in this respect the effect of raising the glucose concentration is the same as the insulin effect. However, beyond the concentration when the glucose polymerizing system is saturated no further increase of oligosaccharides and glycogen occurs, yet insulin produces a specific accelerating effect on oligosaccharide and glycogen

FIG. 7. — Radiochromatogram of aqueous-alcoholic extract of diaphragm muscle of the rat incubated for varying periods with uniformly labelled ^{14}C -glucose in the presence of insulin (Beloff-Chain *et al.*, 1955a).

FIG. 8.—Influence of glucose concentration in medium on the intracellular glucose concentration of isolated diaphragm of the rat (Beloff-Chain *et al.*, 1956b).

TABLE V.—Influence of Glucose Concentration on Distribution of ^{14}C from Uniformly Labelled Glucose in Diaphragm Muscle of Rat*

Glucose Concentration Fraction:	0.1%	0.25%	0.5%	1%
Aqueous-alcoholic extract:				
Zone ps ..	10.5 ± 3.3	21.0 ± 4.7	41.0 ± 3.7	42.0 ± 10.3
.. m ..	3.8 ± 0.6	9.5 ± 1.5	15.5 ± 3.0	17.0 ± 2.6
.. g ..	13.6 ± 1.3	46.5 ± 2.5	121.0 ± 32.0	221.0 ± 22.1
.. l ..	134.2 ± 10.4	202.0 ± 16.2	256.5 ± 31.2	275.0 ± 28.5
Glycogen of insoluble residue				
CO ₂ ..	8.0 ± 2.3	14.0 ± 2.9	16.5 ± 3.0	17.0 ± 4.4
	10.7 ± 8.0	14.0 ± 1.4	15.8 ± 1.7	19.5 ± 2.1

* Results expressed as c.p.m. $\times 10^{-3}$ (counted on paper) per 50 mg. of tissue, wet weight. Mean values of 6 experiments $\pm$ S.E.M. (After Beloff-Chain *et al.*, 1956b.)

synthesis even in the presence of 1% glucose (Fig. 9), again without influencing CO₂ and lactic-acid production. It is therefore clear that the effect on the pattern of glucose metabolism of increasing the glucose concentration is qualitatively and quantitatively different from the effect of insulin. Also, in the adipose tissue an increase of glucose concentration cannot simulate the insulin effect.

The permeability theory originated from experiments on eviscerated animals maintained at normal blood-sugar levels by continuous glucose infusion (Levine, Goldstein, Huddleston, and Klein, 1950). The animals were injected with galactose, a sugar which at that time was thought not to be metabolized by muscle, and it was observed that insulin caused a fall of the blood-galactose concentration to a lower level, which then remained constant. In view of the assumed metabolic inertness of galactose these experiments were interpreted as indicating that insulin had caused an increased transfer from galactose into the muscle—that is, had increased the galactose space. Using radioactive galactose, it was shown (Beloff-Chain *et al.*, 1956b) that galactose actually is metabolized by muscle, though at a low rate, and therefore the basis of the theory is not valid.

However, in view of the particular conditions prevailing in eviscerated animals continuously infused with glucose, we decided to investigate with our quantitative radiochromatographic technique whether insulin did, in fact, stimulate galactose transfer in the isolated diaphragm muscle of the rat, in which it produces such marked stimulating effect on oligosaccharide and glycogen synthesis (Beloff-Chain *et al.*, 1956b). Again, aqueous-alcoholic extracts of rat diaphragms were prepared after incubation with radioactive galactose for different periods. Fig. 10 shows that only one radioactive spot, due to galactose, appears in radioautograms of monodimensional radiochromatograms of such extracts. The figures in Table VI demonstrate that the intracellular concentration of galactose is not affected by insulin after any time of incubation. Therefore, whatever the explanation of the experiments with

FIG. 9.—Radioautograph of chromatogram of aqueous-alcoholic extract of diaphragm muscle of the rat incubated for 90 minutes with 1% glucose, with and without insulin.

eviscerated animals will turn out to be, insulin certainly does not lead to an increase of intracellular galactose in a tissue in which it has a pronounced effect on glycogen and oligosaccharides from glucose.

Apart from these experimental observations, it is difficult to see on thermodynamic grounds how an increase of the concentration of intracellular glucose, which even according to the supporters of the

TABLE VI.—Effect of Insulin on the Intracellular Concentration of Galactose-1- ^{14}C in Isolated Rat Diaphragm*

Time of Incubation	Without Insulin	With Insulin
15 minutes ..	3,310 ± 110 (6)	3,340 ± 130
30 ..	3,420 ± 120 (6)	3,370 ± 110
60 ..	3,200 ± 220 (12)	3,320 ± 190
90 ..	3,140 ± 220 (8)	3,620 ± 400

* Results expressed as radioactivity of galactose c.p.m. per 50 mg. of tissue, wet weight. Diaphragm incubated in O₂ at 37° C. in 0.6 ml. of phosphate saline medium. Galactose concentration in medium, 0.1%. Total radioactivity, 1 μC per Warburg vessel; approximately 7×10^4 c.p.m. (counted on paper). Number of experiments given in parentheses. (After Beloff-Chain *et al.*, 1956b.)

permeability theory can at best be only a small one, can possibly provide the energy required for the marked stimulation of fat by insulin synthesis, observed to occur in adipose tissue (Beloff-Chain *et al.*, 1956a) and the lactating mammary gland, in the latter not only from glucose but also from acetate in the presence of glucose (Balmain, French, and Folley, 1950; Balmain and Folley, 1951).

Experiments to Test the Hexokinase Theory

The fact that increasing the glucose concentration does not produce the same effect on the pattern of glucose metabolism in the rat diaphragm and in the adipose tissue as does insulin really excludes the hexokinase theory of insulin action, for if glucose itself does not simulate the specific effects of insulin, glucose-6-phosphate, believed to be the first product of glucose metabolism, also would not be expected to do so. Furthermore, as it is generally accepted that the muscle aerobically oxidizes glucose, but anaerobically converts it to lactic acid, and as the hexokinase reaction is thought to be involved under both these conditions, it was considered that if insulin acted by stimulating the hexokinase reaction it should prove possible to demonstrate an effect of insulin under anaerobic conditions. A study of the fate of glucose under anaerobic conditions in the diaphragm muscle of the rat was therefore undertaken, using the quantitative radiochromatographic technique referred to above (Beloff-Chain, Catanzaro, Chain, Longinotti, Masi, and Pocchiari, 1959a). The figures in Table VII show that under anaerobic conditions no oligosaccharide and glycogen synthesis took place in the rat diaphragm either in phosphate or on

FIG. 10.—Radioautograph of chromatogram of aqueous-alcoholic extract of diaphragm muscle of the rat incubated for 30 minutes with 0.1% galactose, with and without insulin.

bicarbonate buffer. Hence the reactions which are specifically stimulated by insulin do not take place under anaerobic conditions.

However, a number of interesting new facts regarding anaerobic glucose metabolism were brought to light. Intracellular glucose accumulated under anaerobic conditions irrespective of whether the rat diaphragm was incubated in phosphate or bicarbonate buffer. Contrary to expectation, lactic-acid formation from glucose under anaerobic conditions was not increased, but was often decreased to about half or less than half the aerobic value, again irrespective of the incubation medium. However, when the rat diaphragm was first incubated in oxygen with glucose for a short time and then put in anaerobic conditions, a spurt of lactic-acid production occurred, leading to the formation of more lactic acid than under aerobic incubation conditions for the same period (Table VIII, experiment 1). This spurt was of short duration, lasting for not more than two to three minutes.

When the tissue was incubated under aerobic conditions first with non-radioactive glucose and then radioactive glucose was added after five minutes' anaerobiosis, lactic-acid production was reduced to the normal small amount formed under anaerobic conditions (Table VIII, experiment 3). The presence of glucose in the aerobic phase was necessary. Addition of radioactive glucose after aerobic incubation of rat diaphragm in the absence of glucose did not lead to increased lactate production (Table VIII, experiment 2). A short contact of the tissue with oxygen in the presence of glucose for only three minutes was sufficient to produce the spurt (Table VIII, experiment 4). In fact, the lactic-acid spurt occurred when the glucose was added to the chilled incubation medium and the tissue in the central chamber of Warburg vessels before the equilibration with

nitrogen. Therefore, unless the radioactive glucose is added from the side arm of the Warburg vessel to the tissue after anaerobic conditions have been created, erroneous conclusions may be drawn regarding the intensity of anaerobic glycolysis.

The mechanism of the lactic-acid spurt is not understood, but two possible hypotheses can be suggested. Either some factor in the glycolysis system was irreversibly destroyed by lack of oxygen or some substance (or substances) essential for the conversion of glucose to lactate is (or are) built up anaerobically in diaphragm muscle and is (or are) used up very rapidly under anaerobic conditions. The first, inherently unlikely, hypothesis was disposed of by showing that, after admission of oxygen after 15 minutes' anaerobiosis, all the synthetic and oxidative reactions of glucose function almost normally in the rat diaphragm though aerobic lactic-acid production may be reduced up to a maximum of about 30% (Table IX).

TABLE IX.—Influence of First Incubating Rat-diaphragm Muscle Under Anaerobic Conditions on the Aerobic Metabolism of Glucose and on Oxygen Consumption*

	6 Experiments		14 Experiments	
	Controls, 60 min. in O ₂	Pre-incubated in N ₂ for 5 min., 60 min. in O ₂	Controls, 60 min. in O ₂	Pre-incubated in N ₂ for 15 min., 60 min. in O ₂
Lactic acid ..	49.4±3.2	44.6±5.6	32.5±2.0	20.2±2.0
CO ₂ ..	7.5±0.6	6.1±0.7	5.6±0.3	4.6±0.5
Glycogen and oligosaccharides ..	2.7±0.4	3.1±0.8	1.4±0.2	1.2±0.2
Maltose ..	2.1±0.2	2.3±0.3	1.0±0.1	0.9±0.1
Glucose ..	13.8±0.8	14.3±1.1	20.8±1.1	19.8±0.7
O ₂ uptake (μl.) ..	76.1±3.2	73.8±1.4	83.0±3.8	73.0±3.8

* Results expressed as μg. of glucose converted and μl. of O₂ consumed by 50 mg. of diaphragm muscle (wet weight) incubated in 0.6 ml. of phosphate buffered medium at pH 6.8, at 37° C., glucose concentration 0.1%. Mean values given ±S.E.M. (After Beloff-Chain *et al.*, 1959a.)

TABLE VII.—Aerobic and Anaerobic Glucose Metabolism in Diaphragm Muscle of Rat*

Medium:	Phosphate		Bicarbonate			
	60 min.		15 min.		60 min.	
Time of incubation ..	11		15		7	
No. of rats ..	17		15		7	
.. pieces of diaphragm ..						
Conditions of incubation:	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Lactic acid ..	41.3±4.5	21.0±4.1	21.8±1.3	10.2±1.2	44.5±3.3	16.7±2.2
Glycogen, oligosaccharides, maltose ..	4.6±0.4	Traces	4.2±0.3	Traces	13.7±1.3	Traces
Glucose ..	15.4±0.5	26.4±1.2	11.8±0.9	18.9±1.0	16.0±2.2	32.1±1.6

* Results expressed as μg. of glucose converted per 50 mg. of tissue incubated in 0.6 ml. of phosphate or bicarbonate medium. Glucose concentration 0.1%. Vessel gassed with appropriate gas for 5 minutes. Mean values given ±S.E.M. (After Beloff-Chain *et al.*, 1959a.)

TABLE VIII.—Influence of First Incubating Diaphragm Muscle of the Rat Under Aerobic Conditions on the Anaerobic Formation of Lactic Acid from Glucose, and on Glucose Uptake from the Medium

Experiment No.	Conditions of Incubation	No. of Rats *	No. of Pieces of Diaphragm	Medium	Lactic Acid	Glucose Uptake
1	O ₂ 15 min.	6	6	Bicarbonate	14.3±1.4	52.6±1.5
	O ₂ 15 .. N ₂ 10 min.			Phosphate	37.4±8.3	64.2±5.7
	O ₂ 15 .. N ₂ 10 ..			Phosphate	14.1±2.1	35.9±1.7
2	O ₂ 15 min.	8	8	Bicarbonate	61.4±6.8	76.1±6.6
	O ₂ 15 .. N ₂ 10 ..			Bicarbonate	23.0±1.4	
	O ₂ 15 .. N ₂ 15 ..			Bicarbonate	67.6±5.4	
3	O ₂ 15 min. N ₂ 10 min.	4	8	Bicarbonate	8.1±1.3	
	O ₂ 15 min. N ₂ 15 ..*			Bicarbonate	57.1±2.4	
	O ₂ 15 min. N ₂ 15 ..*			Bicarbonate	6.9±0.6	
4	O ₂ 23 ..	2	2	Phosphate	35.4	
	O ₂ 3 .. N ₂ 15 ..			Phosphate	87.8	
	O ₂ 15 ..			Bicarbonate	24.7±2.9	
5	O ₂ 15 .. N ₂ 5 min.†	4	8	Bicarbonate	52.1±7.5	
	O ₂ 15 ..			Bicarbonate		

* Inert glucose added before the aerobic phase of incubation and glucose-G-¹⁴C before the anaerobic phase.

† In these experiments N₂ was passed for 5 minutes and there was no further incubation in N₂.

Results expressed as μg. of glucose converted to lactic acid per 50 mg. of diaphragm muscle (wet weight) incubated in a bicarbonate or phosphate buffered medium. Mean values given ±S.E.M. Vessels gassed for 5 minutes with the appropriate gas then incubated for periods as indicated below. † Indicates when ¹⁴C-glucose added, following equilibration period with appropriate gas. (After Beloff-Chain *et al.*, 1959a.)

Thus the second explanation remains—that is, the aerobic formation of some product involved in glucose metabolism which is used up anaerobically.

The sum total of glycogen plus oligosaccharides formed from glucose in rat diaphragm after five minutes' aerobic incubation is still much too small to account for the quantity of lactic acid formed on subsequent anaerobiosis. This suggests that the substance (or substances) concerned has a catalytic nature. Work on its identification is in progress.

As there is no insulin effect on lactic-acid formation under aerobic conditions it could be expected that the anaerobic formation of lactic acid could also not be influenced by insulin. This is, in fact, borne out by the experimental evidence, and excludes the hypothesis that insulin acts by accelerating the hexokinase reaction.

Energy-Level Theory of the Mode of Action of Insulin

It is fundamental that any theory on the mode of action of insulin must take into account the basic fact that all reactions stimulated by insulin are of energy-requiring synthetic nature, such as glycogen synthesis from glucose and protein synthesis from amino-acids in muscle, fat synthesis in the lactating mammary gland, and both glycogen and fat synthesis in the brown adipose tissue. These endergonic reactions derive their energy from oxidative processes. It has been previously suggested (Chain, Beloff-Chain, and Pocchiari, 1956) that insulin acts by raising the energy level in susceptible cells. They now consider that, at least in part, this may be achieved by making the TPN-TPNH (triphosphopyridine nucleotide, oxidized or reduced form respectively) system more effective for synthesis.

The TPN-TPNH system is known to be required for fat synthesis (Langdon, 1955, 1957). This system is also involved in the first two steps of the oxidative pathway of glucose metabolism, the so-called pentose shunt—that is, the oxidation of glucose-6-phosphate to phosphogluconate and the oxidative decarboxylation of phosphogluconate to ribulose phosphate and CO_2 . The pentose pathway of glucose oxidation occurs in different tissues to a different extent (Glock and McLean, 1954; Bloom and Stetten, 1955, and Dickens, 1956); and is particularly high in the lactating mammary gland (Abraham, Hirsch, and Chaikoff, 1954) in which a strong insulin effect on the synthesis of fat from acetate in the presence of glucose has been observed (Balmann and Folley, 1951). Abraham, Cady, and Chaikoff (1957), using glucose-1- ^{14}C and glucose-6- ^{14}C , have shown that in this tissue insulin strongly increases the production of CO_2 from glucose-1- ^{14}C (Table X), a finding which suggests that it has shifted glucose oxidation towards the pentose phosphate pathway. A similar result was obtained by Winegrad and Renold (1958) in epididymal adipose tissue (Table XI) in which insulin has been shown to exert a strong accelerating effect on both fat and glycogen synthesis, an effect which was also observed in brown adipose tissue (Beloff-Chain *et al.*, 1956a). From these observations it is quite clear that the TPN-TPNH system is affected by insulin. In view of the fact that fat synthesis and glycogen synthesis are interdependent (Tuerkischer and Wertheimer, 1942; Wertheimer, 1945), it is very probable that the TPN-TPNH system is concerned with both syntheses, and studies to establish its exact

function in glycogen synthesis are at present in progress in our laboratories.

It should be remembered that the hexosemono-phosphate pathway of glucose metabolism which has been shown to be under hormonal control (Glock and McLean, 1955) provides an oxidative mechanism for drawing glucose into the cell and for making available, through the action of the aldolase-ketolase system, an extra supply of hexose phosphates (Fig. 11) which are available for glycogen synthesis provided that the appropriate oxidative phosphorylation processes, possibly TPN-linked, are functioning.

According to this theory there should be in the isolated diaphragm of the rat an increased formation of glucose-1-phosphate and glucose-6-phosphate from glucose in the presence of insulin, but the intermediates of the glycolytic cycle should not be influenced. This is in fact in exact agreement with the finding of Shaw and Stadie (1957), who showed that in the isolated rat diaphragm the formation of glucose-6-phosphate and glucose-1-phosphate is strongly stimulated by insulin

TABLE X.—Conversion of Variouslly Labelled ^{14}C -glucose to $^{14}\text{CO}_2$ and Fatty Acids- ^{14}C by Mammary Gland Slices*

Percentage of Added ^{14}C Recovered as $^{14}\text{CO}_2$				Percentage of Added ^{14}C Recovered as Fatty Acids			
Glucose-1- ^{14}C		Glucose-6- ^{14}C		Glucose-1- ^{14}C		Glucose-6- ^{14}C	
Insulin		Insulin		Insulin		Insulin	
—	+	—	+	—	+	—	+
19.7	35.6	2.8	1.9	4.9	9.6	10.1	23.3

* 500 mg. of slices of mammary gland prepared from lactating rats were incubated in 5 ml. of Krebs-Henseleit bicarbonate buffer pH 7.3, containing 50 μmoles of labelled glucose. 1 unit of glucagon-free insulin was added per vessel. Gas phase, 95% O_2 , 5% CO_2 . Incubation for 3 hours at 37° C. Mean values of 15 experiments given. (After Abraham, Cady, and Chaikoff, 1957.)

TABLE XI.—Effects of Insulin Added In Vitro on the Metabolism of Glucose-1- ^{14}C and Glucose-6- ^{14}C by Adipose Tissue of Rat.* (After Winegrad and Renold, 1958)

Animal No.	Oxidation of Glucose Carbon to CO_2		Incorporation of Glucose Carbon into Lipid	
	Control	+ Insulin	Control	+ Insulin
<i>Glucose-1-^{14}C in All Flasks</i>				
101	0.57	4.06	0.19	2.25
102	0.50	3.72	0.20	1.41
103	0.36	4.87	0.20	2.96
128	0.71	3.92	0.26	1.49
129	0.68	4.69	0.24	2.32
130	0.72	4.01	0.39	1.78
Mean	0.59	4.21	0.25	2.04
<i>Glucose-6-^{14}C in All Flasks</i>				
113	0.17	0.27	0.47	3.82
114	0.13	0.25	0.38	5.31
115	0.20	0.24	0.41	2.97
131	0.14	0.26	0.31	3.55
133	0.25	0.33	0.69	5.57
Mean	0.18	0.27	0.45	4.24

* All values expressed as μmoles of glucose carbon-1 or carbon-6 per mg. of tissue nitrogen. Incubation carried out for 3 hours in Krebs bicarbonate buffer containing 20 mmols per litre of glucose. Lipid refers to ether-soluble lipids. Insulin concentration, when present, 0.1 unit per ml. Tissues paired so as to observe the effects of insulin in tissue from the same animal.

TABLE XII.—Incorporation by Normal Rat Diaphragm In Vitro of Glucose-U- ^{14}C into Glucose-6-phosphate and Glucose-1-phosphate*

Glucose-6-phosphate		Glucose-1-phosphate	
Insulin		Insulin	
0	+	0	+
0.11 $\pm$ 0.02	0.30 $\pm$ 0.12	1.18 $\pm$ 0.96	4.02 $\pm$ 1.07

* Results expressed as μmoles per g. per 2 hours of glucose-6-phosphate and glucose-1-phosphate from glucose-U- ^{14}C . Mean values of 7 and 8 experiments respectively $\pm$ S.E.M. (After Shaw and Stadie, 1957.)

inside the cell (Table XII), but that the formation of fructose-1:6-diphosphate of an intermediate phosphate ester of the glycolytic Embden-Meyerhof pathway occurring on the exterior of the cells and accounting for the major portion of lactic-acid formation is not influenced by insulin, though this compound, when added to the incubation medium, became as strongly ^{14}C -labelled as was the added glucose (Table XIII).

TABLE XIII.—Conversion by Rat Diaphragm In Vitro of Glucose- $U-^{14}\text{C}$ to Fructose-1:6-diphosphate; Non-labelled Fructose-1:6-diphosphate also in Medium (after Shaw and Stadie, 1957)

	c.p.m. per Glucose Equivalent	
	Insulin	
	0	+
F-1, 6-P from medium (re-isolated after 2 hours' equilibration) ..	10,200	10,600
F-1, 6-P from diaphragm ..	0	0
Medium glucose ..	10,600	10,600

Fate of Glucose and Fructose in Brain Tissue

(Beloff-Chain *et al.*, 1955b, 1955c)

I should like to conclude this report on our recent studies on carbohydrate metabolism with a short account of some results pertaining to the metabolism of sugars in brain and pituitary tissue.

Some years ago we showed, using ^{14}C -labelled glucose, that glucose in brain-cortex tissue was converted to a number of amino-acids; glutamic acid, aspartic acid, γ -aminobutyric acid, and alanine were identified (Fig. 12). The presence of free amino-acids in brain tissue has been known for some time, but it was not known that some of them derived from glucose. The question arose of the origin of the nitrogen for the transformation of glucose into amino-acids, a process which was shown to be strictly aerobic and which obviously involved transamination reactions.

FIG. 12.—Radiochromatogram of aqueous-alcoholic extract of rat brain incubated with ^{14}C -labelled glucose. (Two-dimensional chromatogram: $\rightarrow$ butanol:acetic acid:water; $\downarrow$ picric acid:tertiary butyl alcohol:water.) (Beloff-Chain *et al.*, 1955c.)

By incubating brain tissue with ^{15}N -labelled ammonium substrate (Chain, Chiozzotto, Pocchiari, Rossi, and Sandman, 1959a) it could be shown that the source of nitrogen for the transformation of glucose into amino-acids was the ammonium ion. By isolating the amino-acids from a Stein-Moore column their specific activity with regard to ^{15}N and ^{14}C content could be found by determining (1) their total amount by means of the ninhydrin reaction, (2) their radioactivity, and (3) their ^{15}N content after Kjeldahlization by mass spectrophotometry. From Fig. 13 it is evident that the areas of radioactivity and ninhydrin reaction completely coincide in the case of glutamate, aspartate, γ -aminobutyrate, and alanine fractions, whereas glycine and glutamine were only very weakly radioactive.

Mass spectrophotometric determinations of the ^{15}N content of the amino-acid fractions showed that after all the newly formed amino-acids were ^{15}N -labelled, glutamine having the highest ^{15}N content in both the amino and the amido group, the ratio ^{15}N amido nitrogen to ^{14}C being nearly 2 (Fig. 14).

It is evident from these results that glutamine is the first intermediate in the metabolic pathway of the ammonium ion and that the latter is transferred via the amido nitrogen of glutamine. An unexpected result which came to light from these isotope distribution studies was that in alanine and γ -aminobutyric acid the ratio of ^{15}N to ^{14}C was very low

FIG. 11.—Diagram of the pentose phosphate cycle. The overall reaction for one turn of the cycle is: 3-glucose 6-phosphate + 6 TPN + 3 CO_2 + 6 TPNH + glyceraldehyde 3-phosphate + 2-glucose 6-phosphate. [Key to enzymes: (1) Glucose 6-phosphate dehydrogenase and lactonase. (2) 6-phosphogluconate dehydrogenase. (3) Pentose phosphate isomerase. (4) Phosphoketopentose epimerase. (5) Transketolase. (6) Transaldolase. (7) Phosphogluconate isomerase.] After Glock and McLean (1958).

(Fig. 14). If both these amino-acids originated from glutamate, the first through the tricarboxylic acid cycle, the second through the action of glutamic acid decarboxylase, they could be expected to have a $^{15}\text{N}/^{14}\text{C}$ ratio similar to that of glutamic acid. This is, however, not the case, and suggests that these amino-acids may be formed by a different, hitherto not understood metabolic pathway.

Quastel (1959) confirmed the formation of the above-mentioned amino-acids from ^{14}C -glucose, but in addition found the glutamine fraction labelled. As the only important difference between the experimental conditions employed by Quastel and by us was the presence of potassium ions in the incubation medium of Quastel, the effect of these ions on the metabolic pattern of glucose in slices of brain cortex was investigated, always using the quantitative radiochromatographic technique (Chain, Larsson, and Pocchiari, 1959b). The

FIG. 13.—The elution curve of an extract of brain cortex of the rat incubated with glucose- G^{14}C (Chain *et al.*, 1959a).

FIG. 14.—Participation of glucose G^{14}C and $^{15}\text{NH}_3$ in formation of amino-acids, by brain cortex of the rat (Chain *et al.*, 1959a).

results, given in Table XIV, show that the presence of 0.027 M potassium ions stimulated over fourfold the synthesis of glutamine from glucose, and about doubled that of γ -aminobutyric acid, whereas the formation of glutamate, aspartate, and alanine was not significantly affected.

The oxygen uptake and the production of radioactive CO_2 were also higher in the presence of K ions (Table XIV), an effect already noticed by Quastel.

The pattern of fructose metabolism in brain is the same as that of glucose, but fructose is used to a much lesser extent by this tissue than glucose.

In Table XV a quantitative comparison of glucose and fructose metabolism is given, from which it is

TABLE XV.—Metabolism of Glucose and Fructose in Slices of Rat Brain*

Metabolites Formed from Substrates	Substrates	
	Glucose (6)	Fructose (4)
CO_2	55.1 ± 3.8	26.5 ± 2.1
Lactic acid	170.2 ± 19.5	29.6 ± 1.6
Alanine	3.9 ± 0.3	1.7 ± 0.1
Aspartic acid	6.6 ± 0.3	3.8 ± 0.7
Glutamic	23.9 ± 1.9	9.0 ± 0.5
γ -Aminobutyric acid	7.8 ± 0.6	1.3 ± 0.1

* Results expressed as $\mu\text{g.}$ of substrate converted per 50 mg. of tissue (wet weight) after 60 min. in 0.6 ml. of phosphate buffered medium pH 6.8 in O_2 . Substrate concentration 0.1%. Glucose and fructose generally labelled with ^{14}C . 10 μC of radioactive substrate per vessel. Mean values given $\pm$ S.E.M. Number of experiments given in parentheses. (After Beloff-Chain *et al.*, 1955b, 1955c.)

evident that the formation of amino-acids from fructose is nearly three times lower than that from glucose, the formation of lactate five times lower, and the production of CO_2 about one-half.

Fate of Glucose in the Rat Pituitary

(Beloff-Chain *et al.*, 1959b)

In the isolated pituitary gland of the rat glucose is also transformed in free amino-acids; alanine, aspartic acid, glutamic acid and a small quantity of proline were identified, while γ -aminobutyrate was absent (Fig. 15).

Apart from the free amino-acid, radioactivity appeared also in the protein fraction; on hydrolysis of the latter, aspartic acid, glutamic acid, alanine, serine, and proline were identified (Fig. 16). The active protein synthesis from glucose in the pituitary is perhaps connected with the production of hormones by this tissue. In contradistinction to brain tissue, the pituitary formed glycogen and oligosaccharides from glucose. A quantitative comparison of glucose metabolism of the pituitary gland and brain tissue, using the automatic quantitative chromatographic technique, is given in Table XVI. It will be seen that the total utilization of glucose in brain tissue is about twice as high as in

TABLE XIV.—Effect of Potassium and Sodium Ions on Glucose Metabolism and on Oxygen Consumption by Slices of Cortex of Rabbit Brain*

	Alanine	Aspartate	Glutamate	γ -Amino-butyrate	Glutamine	Lactate	CO_2	O_2 ($\mu\text{l.}$)
Na^+ (0.04 M)	1.4 ± 0.3	0.9 ± 0.2	9.8 ± 1.8	1.31 ± 0.3	0.6 ± 0.2	93.9 ± 12.6	19.6 ± 2.2	32.1 ± 1.0
Na^+ (0.017 M) + (K^+ 0.027 M)	1.5 ± 0.4	1.3 ± 0.3	12.2 ± 3.1	2.8 ± 0.9	2.8 ± 1.0	126.2 ± 25.2	26.9 ± 2.8	41.0 ± 3.0
P	0.9	0.4	0.6	0.1	0.05	0.8	0.1	0.01

p considered significant < 0.1.

* The values are expressed as $\mu\text{g.}$ of glucose converted and $\mu\text{l.}$ of oxygen consumed per 25 mg. of tissue (wet weight). Mean values of 5 experiments given $\pm$ S.E.M. (After Chain, Larsson, and Pocchiari, 1959b.)

the pituitary; about three times as much glutamic acid is formed in brain as in pituitary, but the amount of aspartate formed is the same in both tissues and the pituitary appears to synthesize more alanine. It will be an interesting task for the future to study the physiological significance of these differences.

FIG. 15.—Radiochromatogram of aqueous-alcoholic extract of pituitary gland of the rat incubated with uniformly labelled ^{14}C -glucose. (Two dimensional chromatogram—sec. butyl alcohol: formic acid:water; $\downarrow$ phenol:water.) (Beloff-Chain *et al.*, 1959b.)

FIG. 16.—Radiochromatogram of insoluble residue after acid hydrolysis of pituitary gland of the rat incubated with ^{14}C -glucose (Beloff-Chain *et al.*, 1959b.)

TABLE XVI.—Metabolism of Glucose in the Isolated Pituitary Gland of Rat and Slices of Rat Brain*

Fraction	Pituitary Gland	Brain Slices
Alanine	6.7±0.3	3.2±0.1
Aspartic acid	4.7±1.2	4.7±0.5
Glutamic acid	4.0±0.3	14.5±1.0
γ -Aminobutyric acid	None	4.4±0.4
Lactic acid	60.5±6.2	104.5±7.8
CO_2	16.6±2.3	58.0±7.7
Glycogen and oligosaccharides	1.0±0.1	None
Protein, amino-acids	0.9±0.2	"
Total glucose metabolized	94.4±8.4	189.2±12.4

* Results expressed as $\mu\text{g.}$ of glucose converted per 20 mg. of tissue (wet weight) after 3 hours' incubation at 37°C. in 0.4 ml. of phosphate buffered medium pH 6.8 in O_2 . Glucose concentration, 0.1%; total radioactivity, 10 μC per vessel. Mean values of 6 experiments given $\pm\text{S.E.M.}$ (After Beloff-Chain *et al.*, 1959b.)

The findings reported in this survey represent a part of the work on intermediate carbohydrate metabolism which has been in progress in my department during the past ten years.

I hope that I have demonstrated that the new technique of automatic quantitative radiochromatography constitutes a powerful tool for studies on intermediate metabolism and opens up new avenues not

readily accessible by other methods. It is of particular importance for studies connected with the elucidation of the mode of action of drugs and hormones, but should also be of interest to the clinician as a possible tool to obtain, from bioptic samples of tissues, a picture of their functional biochemical state which may eventually prove of considerable value for diagnostic purposes and complement the histological microscopical methods at present used.

REFERENCES

- Abraham, S., Cady, P., and Chaikoff, I. L. (1957). *J. biol. Chem.*, **224**, 955.
 — Hirsch, P. F., and Chaikoff, I. L. (1954). *Ibid.*, **211**, 31.
 Balmain, J. H., and Folley, S. J. (1951). *Biochem. J.*, **49**, 663.
 — French, T. H., and Folley, S. J. (1950). *Nature (Lond.)*, **165**, 807.
 Beloff-Chain, A., Catanzaro, R., Chain, E. B., Masi, I., Pocchiari, F., and Rossi, C. (1955a). *Proc. roy. Soc. B*, **143**, 481.
 — — — — — (1955b). *Biochemistry of Nitrogen*, p. 412. Suomalainen Tiedekatemia, Helsinki.
 — — — — — (1955c). *Proc. roy. Soc. B*, **144**, 22.
 — — — — — (1956a). *Selected Scientific Papers from Istituto Superiore di Sanità, Rome*, **1**, 345.
 — — — — — (1956b). *Ibid.*, **1**, 356.
 — — — — — Longinotti, L., Masi, I., and Pocchiari, F. (1959a). *Ibid.*, **2**, 139.
 — — — — — Masi, I., and Pocchiari, F. (1959b). *Ibid.*, **2**, 109.
 Bloom, B., and Stetten, D., jun. (1955). *J. biol. Chem.*, **212**, 555.
 Chain, E. B., Beloff-Chain, A., and Pocchiari, F. (1956). *Selected Scientific Papers from Istituto Superiore di Sanità, Rome*, **1**, 389.
 — Chiozzotto, M., Pocchiari, F., Rossi, C., and Sandman, R. P. (1959a). In preparation.
 — Frank, M., Pocchiari, F., Rossi, C., Ugolini, F., and Ugolini, G. (1956). *Selected Scientific Papers from Istituto Superiore di Sanità, Rome*, **1**, 241.
 — Larsson, S., and Pocchiari, F. (1959b). In preparation.
 Dickens, F. (1956). *Proc. 3rd int. Congr. Biochem., Brussels*, 1955, p. 170.
 Fink, R. M., Dent, C. E., and Fink, K. (1947). *Nature (Lond.)*, **160**, 801.
 Frank, M., Chain, E. B., Pocchiari, F., and Rossi, C. (1959). *Selected Scientific Papers from Istituto Superiore di Sanità, Rome*, **2**, 75.
 Gemmill, C. L. (1940). *Johns Hopk. Hosp. Bull.*, **66**, 232.
 — (1941). *Ibid.*, **68**, 329.
 Glock, G. E., and McLean, P. (1954). *Biochem. J.*, **56**, 171.
 — — — — — (1955). *Ibid.*, **61**, 390.
 — — — — — (1958). *Proc. roy. Soc. B*, **149**, 354.
 Langdon, R. G. (1955). *J. Amer. chem. Soc.*, **77**, 5190.
 — (1957). *J. biol. Chem.*, **226**, 615.
 Levine, R., Goldstein, M. S., Huddleston, B., and Klein, S. P. (1950). *Amer. J. Physiol.*, **163**, 70.
 Peters, R. A. (1936). *Lancet*, **1**, 1161.
 Quastel, J. H. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, 1958, **3**, 90.
 Shaw, W. N., and Stadie, W. C. (1957). *J. biol. Chem.*, **227**, 115.
 Tuerkischer, E., and Wertheimer, E. (1942). *J. Physiol. (Lond.)*, **100**, 385.
 Villee, C. A., and Hastings, A. B. (1949). *J. biol. Chem.*, **179**, 673.
 Wertheimer, E. (1945). *J. Physiol. (Lond.)*, **103**, 359.
 Winegrad, A. I., and Renold, A. E. (1958). *J. biol. Chem.*, **233**, 273.

Statistics show that a great number of industrial accidents arise from such simple causes as slipping, falling, and striking against fixed objects. Each year much suffering is caused by persons falling over obstructions, tripping on stairs, or coming unintentionally into forceful contact with such things as machines, pieces of plant, stacks of materials, or parts of a building structure. Many of these accidents happen during the night or in the half-light of morning or evening, and many of them can be traced directly to the inadequacy of the artificial light provided. The latest issue of "Accidents—How They Happen and How to Prevent Them" once more draws attention to these all-too-frequent occurrences and emphasizes the importance of good lighting. Docks are among the places where accidents due to bad lighting are apt to occur, and to draw attention to the matter one exhibit in the lighting section of the Industrial Health and Safety Centre at Horseferry Road, Westminster, S.W.1, shows the striking difference between good and bad lighting at a typical dock. (H.M.S.O., No. 40, price 1s. 3d. net.)