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The Metabolism and Functioning of Vitamin-like Compounds

1. AMMONIA FORMATION FROM GLUTAMINE BY HAEMOLYTIC STREPTOCOCCI; ITS RECIPROCAL CONNEXION WITH GLYCOLYSIS

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Nutritional studies have led to the identification of many substances which are essential for normal growth or behaviour of living organisms. In certain but not in all cases, the need for such substances has been found to be connected with their reactions in particular component systems of the organism. The commonest biochemical method of localizing vitamin action has followed Peters's (1936) observation that the metabolism of pyruvate, by intact animals or by tissues separated from them, was retarded during deficiency of aneurin but restored after its addition. This method, involving the successive induction and making good of a nutritional deficiency, has not always been found applicable. This is understandable for, to take as an example the case quoted above, stimulation by aneurin depended on circumstances which included: deficiency in aneurin being compatible with life of the organism, and means being found to produce deficient tissues; the deficient tissues being able to synthesize the added vitamin to the functioning coenzyme; and other components in the system using the coenzyme not being proportionately reduced in deficiency and remaining capable of reacting with the newly synthesized coenzyme.

Investigations of a different type (McIlwain & Hughes, 1944) gave an instance of a relationship which might be regarded as the inverse of that above, and used as a supplementary or complementary method for localizing vitamin action. In this case, the addition of pantothenate to suspensions of bacteria which needed pantothenate in growth was not at first found to initiate or accelerate any process except its own inactivation; but the inactivation was connected with bacterial metabolism in that it occurred only during the progress of a reaction such as glycolysis. Evidence was obtained that this might be due to participation of pantothenate in glycolysis. The effect was thus in some ways comparable to the connexion found between aneurin requirement and carbohydrate intake in animals, and also to certain linked reactions in intermediary metabolism. The investigations of Lwoff & Lwoff (1937), Morel (1941) and of Winzler, Burk & du Vigneaud (1944) are also in many ways analogous.

In the present series an attempt is made to find how general and significant are such reactions and how their occurrence in non-proliferating cells may be connected with the physiological functioning of the substance, for example, in growth. In its relationship to β-haemolytic streptococci, for some strains of which it is a growth-essential (McIlwain, Fildes, Gladstone & Knight, 1939), glutamine has now been found to offer an example of both methods of connexion between a vitamin-like substance and

a metabolic process, in that it was metabolized only during the course of a reaction such as glycolysis; and, also, it stimulated the reaction. The present paper describes the circumstances under which metabolism of glutamine occurs in haemolytic streptococci; the products, kinetics, and specificity of the reaction will be described later.

EXPERIMENTAL

Organisms. The cultures examined are given in Table 1; all were of human origin. β-Haemolytic streptococci, except the two stated as being passaged, were first examined within a few days of their isolation. For the present experiments they were maintained for 2–3 weeks on blood-broth, during which time their reactions to glutamine did not change. From the blood-broth, subcultures on serum-broth-agar were made for daily use. Passage was through mice at intervals of about a month, the cultures meanwhile being maintained by weekly subculture in blood-broth.

Growth. For metabolic studies the organisms were grown for 16–18 hr. in 100 ml. batches of the 'growth medium C' of McIlwain & Hughes (1944). This contained a casein hydrolysate, glucose (0·05 M), arginine (4×10^{-4} M), glutamine (4×10^{-4} M), added ammonium salts ($6{\cdot}8 \times 10^{-4}$ M), several growth-promoting substances, and was buffered with phosphate and $NaHCO_3/CO_2$; its initial pH of 7·6 remained > 6 when the organisms were collected. This was by centrifuging, after which the cells from 100 ml. were washed twice with 10 ml. of 0·9 % NaCl and suspended in salt solution. Suspensions were used within 2 hr. of their preparation, but remained capable of glycolysis and glutamine metabolism after at least 30 hr. storage at 2°.

Metabolism. Most experiments were carried out anaerobically at 37° in Warburg vessels (conical, of c. 20 ml.) with 1–10 mg. dry weight of bacteria and 3–3·5 ml. of solution containing 150 μmol. of $NaHCO_3$ and other reagents in equilibrium with 5 % CO_2 in N_2. Anaerobiosis was maintained by yellow phosphorus in a centre well, and additions of reactants during experiments were made from side-arms. In aerobic experiments either (*a*) the reaction mixture was identical with that above but yellow phosphorus omitted from the well and air-CO_2 used in the gas space, or (*b*) the reaction mixture contained 0·06 M-phosphate of pH 7·6 in place of bicarbonate; the well, 2 N-NaOH; and the gas space, air.

Materials. In most cases specimens of *l*(+)glutamine of natural origin were employed. Commercial specimens were purified by separation of associated materials as Pb salts, precipitation of glutamine as a Hg compound, and its recrystallization from aqueous alcohol (Vickery, Pucher & Clark, 1935). Three independently prepared specimens gave indistinguishable biological responses. Synthetic *dl*-glutamine was prepared from synthetic *dl*-glutamic acid (McIlwain & Richardson, 1939) by Bergmann, Zervas & Salzmann's (1933) method.

Determination of NH_3 *and glutamine.* Free NH_3 was determined in a Parnas apparatus by distillation from a borate-Na_2CO_3 mixture of pH 9 and titration, warm, with N/100 H_2SO_4 in aqueous ethanol, using Tashiri's indicator incorporated in the standard acid according to Conway (1935). Glutamine was determined as the additional NH_3 liberated under the mild conditions of hydrolysis specified by Vickery, Pucher, Clark, Chibnall & Westall (1935); for distillation of the total NH_3 after such hydrolysis, a mixture of NaOH and borate of pH 9·5–10 was used. Both determinations were carried out on measured portions of complete reaction mixtures, without separation of bacteria. It is recognized that caution is needed in interpreting 'labile amide-N' as glutamine, in miscellaneous natural materials; but no inconsistencies have been found in accepting such an interpretation in the present experiments. These involve relatively large quantities of glutamine added as the pure compound, and relatively small quantities of bacterial substance. The 'labile amide-N' from 5 mg. dry weight of bacteria, in a typical experiment in which the NH_3 change due to the organisms was 8 μmol., was 0·5 μmol.; no opinion is expressed with respect to the origin of the bacterial 'amide-N'.

Table 1. *Type and origin of streptococci studied*

Abbreviation used in later tables	Streptococci	Lancefield group (of the β-haemolytic organisms)	Origin
R	*Strep. haemolyticus*	A	National Collection of Type Cultures (no. 5631 'Richards'); passaged through mice
P1	*Strep. haemolyticus*	A	Throat
3081	*Strep. haemolyticus*	A	Vagina
UB	*Strep. haemolyticus*	B	Throat
UC2	*Strep. haemolyticus*	C	Throat
G	*Strep. haemolyticus*	G	Throat; passaged through mice
F	*Strep. faecalis*	(α-Haemolytic)	Faeces

Lactic acid was determined through the acetaldehyde formed on its oxidation (Friedemann & Graeser, 1933) and *succinic acid* by the dehydrogenase of muscle (Krebs, 1937).

RESULTS

A. *Glutamine breakdown by streptococci*

(1) *Its independence of growth and viability.* Preliminary experiments showed that when several strains of haemolytic streptococci were grown overnight in media containing known quantities of glutamine, much of this substance disappeared from the culture fluids and could not be extracted from the bacterial cells, nor detected in them in quantities sufficient to account for the amounts lost from the culture fluids. In attempting to reproduce this process under simpler conditions (Table 2) streptococcal suspensions were added to solutions which contained glutamine and various constituents of the growth media, but which lacked some factors essential for streptococcal growth. It was found that a reaction leading to loss of the labile amide group of glutamine occurred independently of the many constituents of a casein hydrolysate or of a yeast preparation, but required the presence of glucose. The reaction, with washed streptococci, in most cases proceeded readily in inorganic salt solutions containing glucose and glutamine. The reaction required the presence of the organisms, but the suspensions concerned changed little, if at all, in optical density during the reaction. Thus in an experiment in which 8·1 μmol. of glutamine were inactivated in 105 min., the dry weight of streptococci (Richards strain) used was 2·8 mg. This was part of a culture yielding 56 mg. of organisms and originally containing 40 μmol. of glutamine, which was a fivefold excess over the minimum quantity needed for maximal growth of the organisms. The 2·8 mg. of dry bacterial matter were thus associated with 0·4–2 μmol. of glutamine during growth. During the period of metabolism quoted, no change in optical density of the suspensions was observed, which implied any change to be $< 1{\cdot}5\,\%$; the growth of 1·5 % of 2·8 mg. of the organisms would be associated with a change of 6 to 30 mμmol. of glutamine, or 0·07–0·37 % of the change observed. Thus the change cannot be ascribed to growth, and the process of glutamine usage is clearly dissociated from growth. It was also found independent of viability, as actively glycolyzing but mainly non-viable streptococcal suspensions prepared as previously de-

Table 2. *Association of streptococcal metabolism of glutamine with a particular constituent of growth media*

(All reaction vessels initially contained bicarbonate (150 μmol.), glutamine (25–40 μmol.) and the additional substances given below in equilibrium with a gas mixture of 5 % CO_2 in N_2, except in the *aerobic* experiment, when air replaced the N_2. 'Bic.-saline' contained the inorganic salts of Krebs & Eggleston (1940) but with only 3 μmol. of phosphate per vessel. Reactions were initiated by adding the bacterial suspensions to the other reactants.)

Organisms (see Table 1); dry wt. (mg.)	Reaction mixture: Substances (μmol.) in 3·5 ml.	Reaction period (min.)	Change (μmol.) in: Glutamine	Change (μmol.) in: CO_2
R; 3·8	Bic.-saline + glucose (200)	90	− 4·3	+52·2
,,	,, + casein hydrolysate (McIlwain & Hughes, 1944), 20 mg.	90	< 0·2	+ 0·4
,,	,, + yeast preparation (of McIlwain, 1944), 20 mg.	90	< 0·2	+ 3·9
,,	,, + single strength bacteriological infusion broth, 1 ml.	90	+ 0·6	+ 1·7
R; 13·6	Phosphate; $MgSO_4$ (0·5); yeast adenylic acid (0·7); $NaHCO_3$	30	− 0·3	+ 1·0
,,	Phosphate; $MgSO_4$ (0·5); yeast adenylic acid (0·7); $NaHCO_3$ + glucose (10)	30	− 6·2	+72·2
R; 2·5	Bic.-saline + glucose (200)	90	−12·0	+55·2
,,	,, ,, *aerobically*	90	−12·7	(+61·7)
R; 6·9; collected and used at 22 hr.	Bic.-saline	45	− 0·8	+ 1·4
	,, + glucose (200)	45	− 6·7	+58·8
R; 7·4; collected at 22 hr. and used at 46 hr.	,,	45	− 1·1	+ 3·3
	,, + glucose (200)	45	−11·6	+73·3
R; 6·5; collected and used at 46 hr.	,,	45	− 0·5	+ 0·7
	,, + glucose (200)	45	− 1·0	+ 1·6
G; 3·4	,,	70	− 0·3	+ 0·9
,,	,, + glucose (200)	70	− 9·2	+51·0
3081; 5·2	,,	60	− 0·3	+ 0·7
,,	,, + glucose (200)	60	− 4·5	+51·1
UC2; 3·2	,,	54	+ 0·3	< 0·2
,,	,, + glucose (200)	54	− 5·3	+63·0

scribed (McIlwain & Hughes, 1944) reacted with glutamine during glycolysis.

(2) *Accompanying processes: metabolism of glucose.* It was necessary for glutamine metabolism not only that glucose should be present but also that it should itself be metabolized: partial inhibition of glycolysis by iodoacetate, fluoride, or cyanide was found greatly to inhibit the breakdown of glutamine (Table 3). Pantoyltaurine (10 μmol./3·3 ml.) or a penicillin preparation (20 Oxford units/3·3 ml.) affected neither glycolysis nor reaction with glutamine.

carried out on the following basis. If the reaction were due to glucose or its metabolism producing a low E_h and activating an independent enzyme, this might also be achieved by other means. Neither glutathione nor thiolacetate had this effect (Table 3); CN^-, which activates some peptidases, has been observed above to inhibit the glutamine reaction. Concentrations of KCl up to M/10, the effects of which on animal tissues have been interpreted as due to permeability changes, did not induce reaction with glutamine; nor did urethane. Breakdown of the Richards and group G streptococci was obtained

Table 3. *Association of streptococcal metabolism of glutamine, with metabolism of glucose*

(Experiments were performed anaerobically. 'Amino acids' comprised the group of 14 used by Gladstone (1939), in the proportions there stated; other details were as described in Table 2.)

Reaction mixture		Reaction period (min.)	Change (μmol.) in	
Organisms; dry wt. (mg.)	Substances (μmol.) in 3·5 ml.		Glutamine	CO_2
0	Bic.-saline + glucose (200)	70	< 0·2	0
R; 5·4	,, ,,	70	− 9·1	+80·1
,,	,, ,, + iodoacetate (2)	70	− 1·6	+10·9
,,	,, ,, + fluoride (40)	70	− 3·9	+20·7
,,	,, ,, + cyanide (40)	70	− 0·6	+32·3
R	,, ,,	35	−11·0	+52·1
,,	,, ,, + benzene (sat.)	35	− 0·3	+ 4·3
,,	,, ,, + propamidine (25)	35	− 3·9	+30·1
,,	,, ,, + propamidine (2·5)	35	− 8·7	+48·2
R; 4·9	,, + phosphate (200)	105	− 0·3	0
,,	,, + amino-acids, 65 mg.	105	− 1·3	+ 1·1
,,	,, + pyruvate (100)	105	− 0·6	+ 2·0
,,	,, + lactate (100)	105	< 0·2	+ 1·6
,,	,, + glucose (100)	50	− 4·7	+62·0
R; 5·0	,, + glucose (100)	40	− 6·8	+51·0
,,	,, + galactose (100)	140	< 0·2	+ 2·4
R; 2·6	,, 0	145	< 0·3	+ 1·2
,,	,, glucose (200)	70	− 5·1	+54·5
,,	,, sucrose *or* lactose (100)	145	< 0·5	+ 1·4, 1·3
,,	,, maltose (100)	145	− 0·8	+ 2·9
,,	,, mannitol (200)	145	< 0·3	+ 1·1
F; 5·4	,, 0	150	< 0·2	+ 0·6
,,	,, glucose (200)	60	− 3·9	+57·5
,,	,, lactose (100)	150	+ 0·5	+ 0·7
,,	,, sucrose *or* maltose (100)	150	− 0·4	+ 0·6, 0·8
,,	,, mannitol	150	< 0·2	+ 0·5
R	,, 0	105	− 0·7	+ 1·7
,,	,, glucose (150)	65	− 8·5	+55·4
,,	,, KCl (300); or thiolacetate (100 or 100); or glutathione (10)	105	0 to −0·7	+2·2 to 2·8

Replacement of glucose by some structurally or functionally related compounds did not permit the reaction with glutamine (Table 3). Reactions comparable to glycolysis did not take place. Under anaerobic conditions lactate, the main product (see below) of the organisms' reaction with glucose, permitted 1/50 of the glutamine decomposition associated with glucose; the ratio with pyruvate was 1/15.

In attempting to induce the glutamine reaction without metabolism of glucose, experiments were by rubbing with powdered glass by Wiggert, Silverman, Utter & Werkman's (1940) technique and a few variations of it. The resulting preparations were almost inactive towards glutamine.

(3) *Accompanying processes: production of* NH_3. The method used in determining glutamine involved estimation of NH_3 in the same solutions, and it was immediately found that the reaction of streptococci with glucose and glutamine liberated a volatile base, characterized (see later) as NH_3. The anaerobic production per mol. of glutamine undergoing reaction

Table 4. *Yield of ammonia from streptococcal metabolism of glutamine during glycolysis*

(Glutamine was the natural *l*(+) compound except in the instance specified. Details of suspending fluids are given in Table 2; all contained glucose, 200 μmol. per 3·5 ml. Experiments were carried out anaerobically except where indicated. Casein hydrolysate was that of McIlwain & Hughes (1944); 20 mg. were added per 3·5 ml. in the experiment indicated.)

Reaction mixture		Reaction period (min.)	Mean Q_{CO_2} in presence of glutamine	Change (μmol.) in		Ratio mol. NH_3 / mol. glutamine
Organisms; dry wt. (mg.)	Suspending fluid; glutamine added (μmol.)			Glutamine	NH_3	
R; 7·2	Bic.-saline (39·3)	50	320	− 9·2	+ 8·2	0·89
,,	,, (19·7)	50	308	− 8·9	+ 7·5	0·84
,,	,, synthetic *dl*-glutamine (41)	50	306	− 8·7	+ 7·3	0·84
R; 5·2	,, (15·4)	70	212	− 4·4	+ 4·35	0·99
R; 6·5	,, (19·8)	40	503	−11·6	+10·3	0·87
R; 3·4	,, (19·5)	70	403	− 9·2	+ 6·3	0·68
G; 3·35	,, (24·3)	50	446	− 5·2	+ 5·0	0·96
G; 3·5 (organisms kept at 0° 24 hr.)	,, (8·4)	115	39	− 2·05	+ 2·0	0·97
G; 4·2	Bic.-Mg-adenylic acid (20·1)	50	382	− 5·5	+ 5·2	0·94
UB; 6·0	Bic.-saline; casein hydrolysate (23·0)	60	260	− 7·5	+ 7·6	1·01
,,	Bic.-saline; casein hydrolysate (23·0) *aerobically*	60	—	− 4·8	+ 4·9	1·02
,,	Bic.-saline only, *aerobically* (23·0)	60	—	− 7·3	+ 3·8	0·52
UC2; 6·8	Bic.-saline (19)	50	263	− 3·7	+ 3·6	0·97
P1; 5·5	,, (23·6)	50	280	− 3·6	+ 3·4	0·95
P1; 4·25	,, (23·5)	65	294	− 6·8	+ 5·8	0·85
3081; 5·2	,, (22·9)	60	261	− 4·7	+ 4·9	1·04

approximated to 1 mol., the commonest yield being between 0·85 and 0·97 mol./mol. of glutamine (Table 4). Lower ratios were found occasionally, especially aerobically (in the phosphate medium described in the experimental part), but ratios significantly greater than unity were not observed. Ratios determined anaerobically were not markedly dependent on the strain of organism, its Q_{CO_2}, or the suspending fluid.

B. *The anaerobic reaction of streptococci with glucose*

For examining its products, the reaction was studied under the conditions employed in following the organisms' reaction with glutamine: anaerobically, with washed suspensions in saline solutions containing inorganic salts and bicarbonate which, with the CO_2 of the gas phase, formed a buffer of pH 7·6. The effects of additional substances, and the course of the reaction, are considered later. In the simple medium, with excess glucose, the majority of the strains caused a gas evolution of 200–400 μl./mg. dry weight/hr. (Q_{CO_2}; Tables 4, 7, 9). With limited quantities of glucose, gas evolution by the two strains examined was found to proceed at rates almost identical with those given in the presence of excess glucose, but to reach sharply a maximum value corresponding to a little less than 2 mol. of gas per mol. of glucose added (Table 5).

Table 5. *Products of streptococcal glycolysis*

(Reactions were carried out anaerobically in the bicarbonate saline of Table 2.)

Reaction mixture		Gas evolved from bicarbonate						Lactic acid	
		Volume (μmol.) at times (min.)					Ratio mol. gas / mol. glucose		Ratio mol./mol. glucose
Organisms dry wt. (mg.)	Glucose (μmol.)	*8*	*18*	*28*	*38*	*58*		μmol.	
G; 6·1	0	0·2	0·7	0·8	0·9	1·1	—	0·5	—
,,	10	7·3	16·4	19·0	19·2	19·3	1·93	19·0	1·90
,,	15	7·6	16·9	25·8	29·3	29·8	1·98	28·2	1·88
,,	25	7·4	17·3	26·9	35·2	48·0	1·92	47·8	1·91
		Volume (μmol.) at times (min.)							
		15	*35*	*50*	*65*				
R; 1·9	0	0·5	0·6	0·6	0·7		—	—	—
,,	10	5·3	13·9	19·0	19·1		1·91	—	—
R; 2·6	200	11·1	22·9	31·6	39·6		—	38·9	—

Table 6. *Source of gas evolved by streptococci during glycolysis in the presence of bicarbonate*

(Vessels contained in their main portion the saline of Table 2 (but with the lesser quantities of bicarbonate specified below), glucose, 200 μmol., and other substances indicated. Organisms were contained in one side-arm and 0·2 ml. 5N-H_2SO_4 in another. Experiments, except where indicated, were anaerobic, and were continued until gas production by the organisms themselves was maximal.)

Reaction mixture		Total gas (μl.) evolved from duplicate vessels		Period of reaction before adding acid to 2nd vessel (min.)	Difference in gas evolution between (*a*) and (*b*), as % of (*a*)
Organisms	Solution	(*a*) By addition of acid before reaction	(*b*) During reaction and by the later addition of acid		
G	No addition	331	325	62	−1·8
„	With phosphate, 400 μmol.	158	162	80	+2·5
„	With casein hydrolysate	274	280	85	+2·2
„	No addition; *aerobic*	299	298	95	<1
R	No addition	335	329	62	−1·8

By determining the total CO_2 which could be liberated by excess acid from the complete system of organisms and reagents, before and after a period of metabolism (Table 6), the gas production was shown to be almost entirely due to displacement of CO_2 from bicarbonate by acids formed from the bacteria. The same was found true aerobically. It was anticipated (cf. Hewitt, 1932) that the anaerobic product from glucose would be lactic acid, and this was determined (Table 5). Results showed that lactic acid appeared in the ratio of about 1·9 mol./mol. of glucose supplied, i.e. that all but 5 % of the glucose which reacted and 1 or 2 % of the gas which was produced could be accounted for by the glycolytic reaction: $C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$. Of other possible products, succinic acid has been determined and any production found to be $<0·5$ mol. % of the glucose reacting. Evolution of CO_2 from bicarbonate- and glucose-containing solutions by the present streptococci is subsequently referred to as a measure of glycolysis.

C. *Stimulation of glycolysis by glutamine*

(1) *Suboptimal glycolysis of streptococcal suspensions.* The rates of glycolysis by β-haemolytic streptococci, in the relatively simple media of the preceding experiments, were not always constant throughout the course of the reaction. On its initiation by addition of streptococcal suspensions to the glucose-containing solutions, the reaction began at a rate which, in the course of some 30 min., fell to about 70–80 % of its initial value; after this it remained approximately constant or fell at a slow rate. Fig. 1 illustrates this with respect to the Richards strain; Table 7 includes other strains of streptococci, of which the majority behaved similarly. The rate of glycolysis was little affected by the addition of many pure substances (see below); it was increased some 5–15 % by a casein hydrolysate or a yeast preparation (Table 7), and was considerably increased by the presence of broth. Suspensions which had not been washed in saline also afforded higher values.

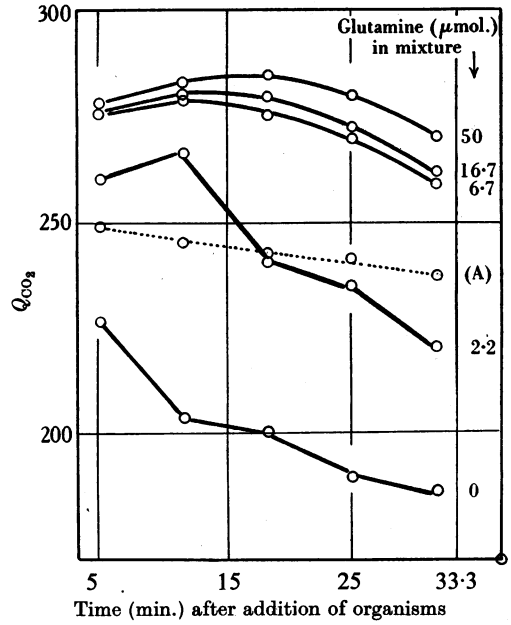

Fig. 1. Effect of glutamine on streptococcal glycolysis. Ordinate: Anaerobic gas evolution in successive periods of 10 min., expressed as Q_{CO_2} (μl./mg. dry wt./hr.) from suspensions of the Richards strain after addition to saline-bicarbonate-glucose containing the quantities of glutamine indicated (except the dotted line *A*, which refers to a mixture containing no glutamine but NH_4Cl, 50 μmol.). When the experiment was stopped 35 min. after addition of the organisms, the following quantities (μmol.) of glutamine remained: 43·3 (of 50), 10·2 (of 16·7), 0·1 (of 6·7) and <0·1 (of 2·2).

The organisms were thus capable of glycolysis at rates some 60–100 % higher than those observed in simple salt solutions, and substances inducing higher

Table 7. *Effects of materials on the rate of streptococcal glycolysis*

(Saline suspensions of organisms were added from side-arms, to vessels containing bicarbonate-saline with glucose (200 μmol.) and the addenda specified below, at times taken as zero. Details of constituents of the reaction-mixtures are given in Table 2. In cases marked (*) observation was prevented by growth of the bacteria under examination. No allowance has been made for CO_2 retention and the stimulation recorded as being given by broth and serum solutions is therefore minimal.)

Reaction mixture		Q_{CO_2} as % of the 5–25 min. value without addenda (which is given italicized, in parentheses, as μl./mg. dry wt./hr.)	
Organisms; batch	Addenda	5–25 min.	45–65 min.
R; I	0	(*256*)	78
„	Ox serum, 29%	105	106
„	Ox serum, 5·7%	97	104
„	Broth, 29%	159	*
„	Glutamine, 0·007 M	141	135
R; II	0; washed	(*295*)	81
„	0; not washed	196	*
R; III	0	(*261*)	81
„	Casein hydrolysate (10 mg.) and yeast preparation (10 mg.)	110	89
„	Yeast preparation and glutamine, 0·007 M	149	141
„	Glutamine, 0·007 M	145	135
P1	0	(*223*)	71
„	Casein hydrolysate (20 mg.)	105	88
„	Casein hydrolysate and glutamine 0·007 M	129	127
„	Broth, 29%	158	127
„	Broth, 29% and glutamine 0·007 M	155	125
3081	0	(*200*)	105
„	Casein hydrolysate (10 mg.) and yeast preparation (10 mg.)	118	123
„	Serum, 29%	103	110
„	Broth, 29%	163	*
„	Glutamine, 0·007 M	124	136
UC2	0	(*460*)	85
„	Serum, 29%, 5·7%	93, 95	—
„	Broth, 29%	163	—
„	Glutamine, 0·007 M	100	112

rates were present in broth and perhaps removed from the cells by washing.

Glutamine made good a large part of the organisms' deficiency in this respect. Glycolysis was increased to up to 100% of its original value, the increase varying with different strains and preparations of the streptococci. With the strains of Table 7, the increase in glycolysis due to glutamine was not additive to that caused by broth, though greater stimulation was found to be caused by casein and yeast preparations with glutamine, than by either alone. The present broth permitted growth of exacting streptococci in glutamine-deficient media (cf. McIlwain *et al.* 1939). It was concluded that the stimulation caused by broth was due in part to glutamine, and in part to substances of the sort occurring in the casein hydrolysate or yeast preparation. Fig. 1 shows 0·003 M-glutamine to be adequate for its maximal effect with the present experimental arrangement; the stimulation given by 0·00063 M-solutions, though initially not much less than that given by 0·015 M, fell much more rapidly.

As the experiments of Fig. 1 were carried out with organisms whose glycolysis was falling in the initial phase of the reaction, a different experimental arrangement was chosen to show more clearly whether the effect of glutamine was a stimulation of glycolysis rather than its maintenance by the substance at a level from which it fell in its absence. In Fig. 2A glutamine was added to already glycolyzing organisms at different times during the reaction. In each case a large and prompt increase in glycolysis followed. The substance is thus capable of increasing, and not only of maintaining, glycolysis. Its effect is manifested within 2 min. of its addition and is thus not likely to be due to growth of the bacteria; absence of growth was confirmed by optical density measurements.

(2) *Possible impurities in glutamine specimens.* The majority of the present experiments had been carried out with specimens of natural *l*-glutamine, which was shown by isolation (McIlwain *et al.* 1939) to be the form encountered by the organisms in animal tissues. As the glycolysis experiments were carried out in simple media, it was of first importance to find whether the stimulation caused by the specimens was due to glutamine rather than to biologically active impurities which can always be assumed to be associated with such preparations. Carefully purified natural glutamine was found to give the same response as ordinary specimens, and, further, glutamine synthesized from synthetic glutamic acid gave a similar response. The quantitative considerations of the following paragraphs give further reasons for associating the effect on glycolysis with glutamine itself.

(3) *Responses to varying concentrations of glutamine.* In the experiment of Fig. 2B, the quantities of glutamine added increased the total glycolysis in the first hour by amounts between 67 and 42% of that of the control without glutamine. Nevertheless, the maximum increases in Q_{CO_2}, which occurred in the first 5 or 10 min. after glutamine had been added, were approximately equal, independently of the quantity of glutamine added (0·9–45 μmol.) and were all of about 90%. The effect of falling quantities of glutamine was to produce a more rapid fall

in the rate of glycolysis during the hour after their addition.

A reason for this behaviour is to be seen in the finding of § A, that glutamine is decomposed during glycolysis. At the end of the experiment of Fig. 2B, little or no glutamine remained of the smallest quantity added and only about one-third of the 4·5 μmol. A similar decomposition is shown to have occurred during the differently arranged experiment of Fig. 1. It will be seen that in both these cases, when all added glutamine had been decomposed, CO_2 evolution in the presence of glutamine was due to the same processes as occurred in its absence, its main product, lactic acid, was estimated in solutions in which glycolysis had occurred with and without glutamine. It was found (Table 8) that the increased gas formation could largely be accounted for by increased lactic acid production. Succinic acid formation was found to remain at $<0{\cdot}5$ mol. %. Anaerobic CO_2 evolution by streptococci with glutamine as their only substrate, remained at the low level associated with the 'endogenous' metabolism

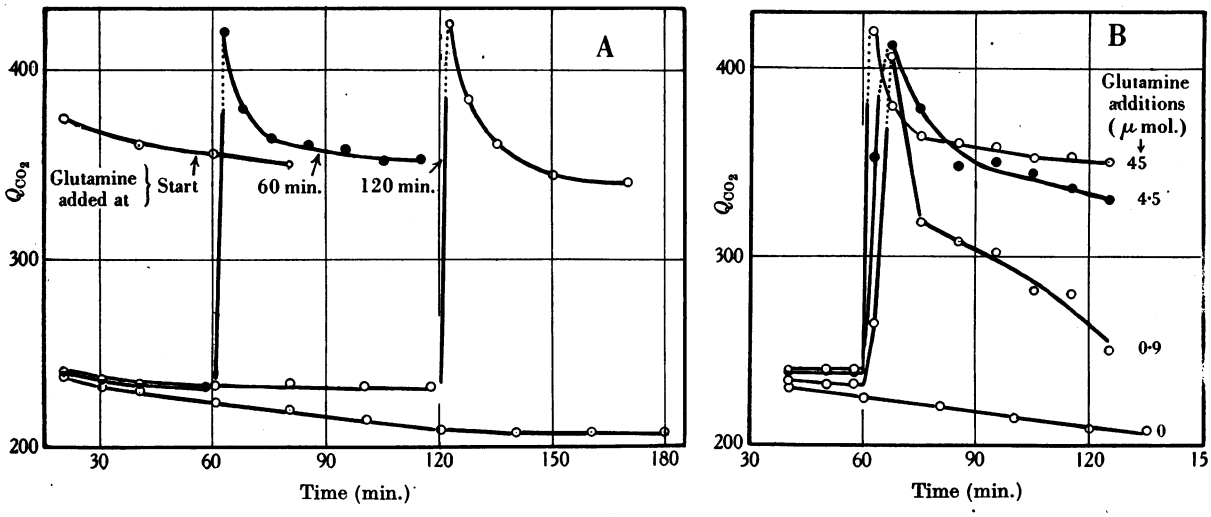

Fig. 2. Promptness of response by glycolyzing streptococci to additions of glutamine. Units: as Fig. 1. Vessels initi contained saline-bicarbonate-glucose and organisms in 3 ml., except the controls, of which the volume was 3·5 ml. In A, glutamine (45 μmol. in 0·5 ml.) was added at the times indicated; in B, the varying quantities of glutamine shown (in 0·5 ml. water) were added at 60 min. After the latter experiment, the quantities of glutamine (μmol.) remaining were: 36·2 (of 45), 1·6 (of 4·5) and $<0{\cdot}1$ (of 0·9).

the level of glycolysis had not fallen to that of the control without added glutamine; this is considered below.

(4) *Products of glucose metabolism in the presence of glutamine.* To see whether the increased rate of of the organism (Tables 2 and 3). The final total of gas evolved from limited quantities of glucose in experiments of the type of Table 6 but with added glutamine, differed from controls without glutamine by <2 %.

Table 8. *Comparison of increase in gas evolution from bicarbonate-glucose, following addition of glutamine or ammonia, and increase in lactic acid formation*

(Vessels contained excess glucose (200 μmol.) and the reagents in the saline-bicarbonate of Table 2. Suspensions of the Richards streptococci were added from side-arms, and the reaction occupied 110 min.)

Addendum (μmol.)	Gas evolution	Lactic acid formed
0	51·3 μmol. (A)	49 μmol. (B)
NH_4Cl (40)	116 % of (A)	119 % of (B)
NH_4Cl (40) and glutamine (10)	139 % of (A)	140 % of (B)
Glutamine (10)	135 % of (A)	136 % of (B)

D. *Actions on glycolysis of some compounds functionally or structurally related to glutamine*

The effects of the following compounds are, for present purposes, selected from a fuller investigation of the structural and species specificity of glutamine-action.

(1) *Ammonia.* It had been noticed in several instances, of which Figs. 1 and 2 give examples, that although optimal stimulation of glycolysis in the presence of small quantities of glutamine was transitory only, the rate of glycolysis did not, when practically all added glutamine had been decomposed, fall to the level of controls without added glutamine. As ammonia was known to be formed

from glutamine during such experiments (§ A) and known to stimulate reactions in yeast which are analogous to the present one (Winzler *et al.* 1944), the effect of ammonium salts on streptococcal glycolysis was examined. An increase was found which was sufficient to account for the level of glycolysis after decomposition of glutamine.

Table 9. *Stimulation of streptococcal glycolysis by ammonia; compared with, and in addition to, the effect of glutamine*

(Experimental arrangement as described in Table 7.)

Reaction mixture		Q_{CO_2} as % of the values for the same period without addenda (italicized, in parentheses; as μl./mg. dry wt./hr.)	
Organism; batch	Addenda (μmol.)	5–35 min.	35–65 min.
R; I and II	0	(*209, 327*)	—
„	NH_4Cl, 0·05	100, 100	—
„	NH_4Cl, 0·5	104, 108	—
„	NH_4Cl, 5	116, 119	—
„	NH_4Cl, 50	122, 119	—
„	Glutamine, 25	143, 137	—
R; III	0	(*292*)	(*280*)
„	NH_4Cl, 2	109	105
„	NH_4Cl, 40	115	106
„	Glutamine, 10	135	134
„	Glutamine, 10 and NH_4Cl, 2	136	136
„	Glutamine, 10 and NH_4Cl, 40	138	133
R; IV	0	(*449*)	(*427*)
„	NH_4Cl, 25	116	117
„	KCl, 25	98	102
„	KCl, 300	111	108
Strain F	0	(*190*)	—
„	NH_4Cl, 25	101	—
„	Glutamine, 25	112	—
„	Glutamine, 25 and NH_4Cl, 25	113	—
UC2	0	(*305*)	(*295*)
„	NH_4 glutamate, 10	120	121
„	Glutamine, 10	162	163
UB	0	(*201*)	(*181*)
„	NH_4 glutamate, 25	117	119
„	Glutamine, 25	133	135

The increase in glycolysis caused by ammonium salts (Table 9) was not sufficiently great to suggest that the major effect of glutamine could be due merely to the ammonia derived from it. The increase by ammonium salts was less in magnitude even when concentrations of the salts were used which were much greater than could be produced in the medium as a whole, from the glutamine concentrations having maximal effect. Thus, during the experiment of Table 9 with batch I of the Richards streptococci, the glutamine decomposed after a period of 50 min. glycolysis was 3·8 μmol. and the ammonia of this reaction mixture, 3·0 μmol.; yet the stimulation of glycolysis caused by 50 μmol. of added ammonium chloride, was just half of that caused by the glutamine. Other results quoted in Table 9 indicate that such considerations are not vitiated by zone effects in the actions of different concentrations of ammonium salts; the possibility of ammonia exerting a greater or different effect when produced locally by the organism itself is considered below.

Nevertheless, the effect of ammonium salts on glycolysis did not appear to be distinct in nature from that of glutamine; the actions of the two compounds were additive if at all, to a small extent only (Table 9) and like glutamine, ammonium salts increased lactic acid formation from glucose in proportion to the increase caused in CO_2 displacement from bicarbonate (Table 8).

Certain effects of K salts on glycolysis in animal tissues have been found similar to those of NH_3 (Weil-Malherbe, 1936). Streptococcal glycolysis was found to be stimulated by high concentrations of KCl; a 0·1 M-solution had less effect than 0·005 or 0·001 M-NH_3 (Table 9).

(2) *Biotin.* As Winzler *et al.* (1944) found an action of biotin on yeast respiration and fermentation to be intimately connected with the action of ammonium salts on the same process, the effect of biotin on streptococcal glycolysis in the presence and absence of ammonia, was examined. In neither case did biotin have an observable effect. In comparing this result with the action on yeast, it is to be noted that although the reagents of the present experiments were biotin-free, no attempt was made to obtain streptococcal cells deficient in biotin.

(3) *Arginine and urea.* Of the compounds related to glutamine which have been studied, many are potential sources of ammonia to various bacteria; but during glycolysis the present streptococci produced ammonia at a considerable rate only from arginine (Table 10). This reaction differed notably from that with glutamine in being independent of glycolysis; it was presumably due to the action of arginine dihydrolase which Hills (1940) found to be exhibited by suspensions of streptococci, including the Richards strain.

Comparison of the effects of arginine and glutamine on glycolysis as measured by CO_2 evolution, required knowledge of the effects of the compounds and of changes occurring in them, on the CO_2-$NaHCO_3$ equilibrium during manometry. The buffering action of the concentrations of arginine, glutamine and α-ketoglutarate of Table 10 resulted in a small binding of CO_2. This was assessed by the method of Dickens & Simer (1932) over the range of acid formation of the experiments of Table 10, and would affect the values there quoted by $< 5\%$. In addition, however, 1 mol. of CO_2 is fixed by each

Table 10. *Stimulation of streptococcal glycolysis by arginine and urea; absence of comparable effect by α-ketoglutarate*

(Experimental arrangement as described in Table 7, using the Richards streptococci (different batches in A, B and C).)

	Compound added (μmol.)	Q_{CO_2} as % of the value without addendum, during the first hour (italicized, in parentheses; as μl./mg. dry wt./hr.) 0–60 min.	60–120 min.	Mean Q_{NH_3} (μmol./mg. dry wt./hr.)
A	0	(*219*)	83	<0·03
	NH_4Cl (10)	125	124	—
	Arginine (10)	110	114	2·91
	„ corrected as described in text	125	128	—
	Urea (10)	108	118	<0·03
	„ (2)	105	108	<0·03
B	0	(*247*)	80	—
	α-Ketoglutarate (10)	95	98	—
	„ (2·5)	102	98	—
	NH_4Cl (25)	122	103	—
	„ and α-ketoglutarate (10)	125	100	—
	„ „ (2·5)	122	97	—
	Glutamine (10)	151	143	—
		Lactic acid produced during 90 min., as % of the value without addition (italicized, in parentheses; as μmol.)		
C	0	(*43·5*)		<0·03
	NH_4Cl (10)	125		—
	„ (2·5)	120		—
	Arginine (10)	128		3·2
	„ (2·5)	121		1·8
	Glutamine (10)	171		1·65

mol. of arginine decomposing according to the reaction (Hills, 1940):

$$H_2CO_3 + R.NH.C(NH)NH_2.H_2CO_3 + 3H_2O \rightarrow R.NH_2.H_2CO_3 + 2NH_4.HCO_3.$$

As the increase in bicarbonate ion following this reaction is small in comparison with the quantity added as $NaHCO_3$, an approximate correction for the CO_2 fixed could be made by adding to the observed gas evolution during arginine decomposition, 1 mol. of CO_2 for each 2 mol. of NH_3 formed. In this way the corrected values of Table 10 were obtained. They indicated that the effect of arginine on glycolysis was similar to that of ammonium salts. The validity of this conclusion was confirmed by determining iodometrically the lactic acid formed in similar experiments, with the results given in C, Table 10.

The rate of ammonia production from arginine by the streptococci, expressed as a mean Q_{NH_3} in Table 10, was equal to or greater than their rate of production of ammonia from glutamine during glycolysis. If the effect of glutamine on glycolysis were due only to ammonia, produced in proximity to the organisms, its effect would not be expected to be greater than that of arginine.

Urea also stimulated glycolysis by the Richards streptococci (Table 10), but ammonia was not produced from it in notable quantities; production at a rate 1/100 that of the liberation from arginine could have been detected. The stimulation was less than that by ammonia.

The possible production of glutamine from urea and arginine was also examined, but $< 0{\cdot}3\,\mu$mol. was found to be produced by streptococci from 25 μmol. of either substance, with or without glucose, during a time in which $8{\cdot}3\,\mu$mol. of NH_3 were liberated from the arginine. A possible interpretation of the organisms' response to urea is that small quantities of ammonia were produced from it and underwent further reactions with the organisms; small quantities of added ammonia have been observed to disappear from certain reaction-mixtures during glycolysis.

(4) *Glutamate and α-ketoglutarate.* Sodium glutamate (5 or 50 μmol./3·5 ml. of glucose-bicarbonate saline) was found to be without effect on the anaerobic glycolysis of the Richards and Group G streptococci. The stimulation given by ammonium glutamate was not greater than that given by ammonium chloride; Table 9 includes results with other organisms which showed that ammonium

glutamate did not have the effect of glutamine. Ammonium glutamate was also present in the casein hydrolysate, whose stimulation of glycolysis (Table 7) was much less than that of glutamine.

α-Ketoglutarate alone had a small effect only on glycolysis. A range of concentrations, in the presence of ammonium salts, had little effect other than that of the salts themselves; in addition to the results given in Table 10, this has been found to be the case with other preparations of Richards streptococci of Q_{CO_2} 377 and 264 μl./mg. dry weight/hr. and mean Q_{HN_3} (glutamine) of 2·04 and 1·16 μmol./mg. dry weight/hr. respectively, whose glycolysis was stimulated by glutamine to 147 and 139% of the control values, during the first 30 min.

DISCUSSION

The present findings appear to offer the first instance in which relatively small quantities of glutamine specifically (as distinct from α-ketoglutarate or glutamate) have been observed to stimulate a major metabolic process which proceeded, though more slowly, in the absence of added glutamine. A conclusion of the type commonly drawn from such evidence would be that glutamine normally functioned in the process of glycolysis which it stimulated. This is feasible in the present instance, as the increased glycolysis was not beyond that obtainable in mixtures of naturally occurring substances which the organisms ordinarily encounter; the streptococcal cells normally contained material with some of the chemical properties of glutamine, and its behaviour as a growth essential showed glutamine to subserve, specifically, some function of importance to the cells. The effect on glycolysis was thus less likely to be an artefact than that given, for example, by arsenites or nitrophenols.

There is in addition the less common and independent type of observation, that glutamine reacted, with loss of NH_3, during its service as growth-factor and also during the course of glycolysis in non-proliferating streptococcal suspensions. Streptococci have not been found to cause changes in glutamine under other circumstances (cf. also Hills, 1940). Processes have been examined which might liberate, activate or facilitate access to an independent enzyme. These included physical breakage of the bacterial cells; the addition of benzene, K salts, thiol compounds and miscellaneous natural materials. Of such treatments, those which prevented glycolysis prevented also the glutamine reaction. Inhibitors showed similar effects, but it was noticeable (Table 3) that the proportional inhibitions caused in glycolysis and glutamine breakdown were usually not the same. This suggests that one may expect to separate the glutamine reaction from the over-all process of glycolysis, possibly through study of the separate reactions which constitute it; but that metabolism of glutamine is firmly associated with that of glucose in the ordinary behaviour of streptococci.

Thus two lines of evidence associate glutamine with glycolysis in streptococci. Previous studies of the compound's metabolic significance have concerned organisms more complex than bacteria. In certain plants, where it constitutes a much greater proportion of the dry weight than it does of streptococci, glutamine has been considered to be a non-toxic reserve of NH_3 or α-ketoglutarate. The known importance of the latter compound in carbohydrate metabolism has been offered in explanation of interrelations observed in plants between glutamine and carbohydrates (Chibnall, 1939). Glutamine was not in this case allocated a catalytic role, except in so far as the α-ketoglutarate formed from it might function in cycles of the *iso*citrate type. The conclusion thus differs from that derived above with respect to streptococci.

Uncombined glutamine is widely distributed in animal tissues (McIlwain *et al.* 1939; Hamilton, 1942; Harris, Roth & Harris, 1943; Archibald, 1944) and hydrolysis to glutamic acid and ammonia has been found to occur in several tissues. The reaction in liver and kidney (Krebs, 1935; Archibald, 1944) differed from that in streptococci in being independent of added carbohydrate and in not being inhibited, but sometimes stimulated, by CN^-. Other aspects of the behaviour of glutamine in animal tissues showed some points of resemblance—possibly superficial—to the streptococcal reaction. Thus the concentration of a glutamine-like substance in human blood fell after administration of glucose (Harris *et al.* 1943). Respiration of certain tissues which synthesized glutamine from added glutamic acid was accelerated during the synthesis (Krebs, 1935; Weil-Malherbe, 1936); but in this case glutamic acid itself was oxidized. Glutamine synthesis was found in these investigations to take place under certain limited conditions: only aerobically in kidney, whose reaction with carbohydrate was aerobic only; in brain and retina, anaerobically also, as could glycolysis; CN^- inhibited the synthesis and respiration in kidney but neither glycolysis nor the synthesis in retina. These limitations were interpreted (Krebs, 1935) as due to requirement of energy for glutamine synthesis; but they are very similar to the circumstances under which NH_3 is produced from glutamine in the present experiments.

Further experiments are required to specify the association between glutamine and streptococcal glycolysis, but the following observations are suggestive. Weil-Malherbe (1936) confirmed Krebs's (1935) finding that the NH_3 reacting in animal tissues with glutamic acid not only formed glutamine but was transferred further, and considered such

reactions to be of functional importance. In streptococci the greater part of the labile amide of glutamine appeared as NH_3. The general view regarding such linked metabolism of vitamin-like compounds, derived from previous studies of the behaviour of pantothenate, has been that they represent an unbalanced performance of a normal process, disturbed possibly through presentation of a relatively large quantity of the substance concerned. The transference of NH_3 rather than its liberation may thus be the significant reaction of glutamine which is linked with streptococcal glycolysis. The stages of conversion of glucose to lactic acid in certain bacteria and in animal tissues are closely similar and the possible participation of NH_3 transference in reactions associated with glycolysis in animal tissues has been suspected but not found to be associated with glutamine (cf. Kleinzeller, 1942). Reasons can, however, be seen for streptococci affording a specially suitable material for showing such association: intact organisms can be used; their requirement for glutamine as growth factor indicates a relevant deficiency, and their high rate of glycolysis is associated with little endogenous change in carbohydrate or NH_3.

SUMMARY

1. Glutamine (determined as labile amide-ammonia) disappeared during growth of several strains of haemolytic streptococci in a complex medium. The process could be reproduced in mixtures of washed non-proliferating streptococci with media constituents, when it occurred at rates of $-0{\cdot}5$ to $-2\,\mu$mol. glutamine/mg. dry weight of organisms/hr.

2. Reaction did not occur between glutamine and streptococci in salt solutions; the medium constituent necessary to the reaction was identified as glucose. It was necessary for glutamine metabolism under a wide variety of experimental arrangements, not only that glucose should be present but also that it should itself be metabolized.

3. The over-all reaction of streptococci with glucose, in the presence or absence of glutamine, was a glycolysis with conversion of 95% of the glucose to lactic acid. The rate of this process in washed suspensions was increased by various media constituents, and taken to up to 190% of its original rate, by the addition of glutamine. Such stimulation by glutamine began within 2 min. of its addition and fell, as glutamine was decomposed, to a level which, when glutamine could no longer be detected, nevertheless remained above that of a control without added glutamine.

4. The organisms' reaction with glutamine produced a maximum of 1 mol. of a volatile base per mol. of glutamine reacting. Their glycolysis was stimulated also by NH_3, but to a lesser degree than by glutamine. Of related compounds, none gave a stimulation equal to or greater than NH_3, but the effect of arginine approached that of NH_3; NH_3 was produced from arginine by the organisms, independently of glycolysis. Stimulation of glycolysis by NH_3 appeared adequate to account for the effects of arginine and of urea (which also accelerated glycolysis) and also for the after-effect of glutamine.

5. It is considered that glutamine itself, as distinct from α-ketoglutarate or glutamate, plays a role in streptococcal glycolysis; with evidence from the behaviour of glutamine and NH_3 in other types of organism, it is suggested that this participation may take the form of an NH_3 transference.

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