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The Metabolism of Acetaldehyde by the Brain *in vivo*

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(*Received* 30 *November* 1962)

The concentrations of certain metabolites in the rat brain are profoundly affected by the physiological state of the animal (Richter & Dawson, 1948; Richter & Crossland, 1949; Dawson & Richter, 1950). In particular, stress (induced by gentle tumbling) is accompanied by an increase in the concentrations of lactate and phosphocreatine, and a decrease in that of acetylcholine. It was decided to extend this work and to ascertain whether these biochemical effects of stress on the central nervous system were modified by the ingestion of ethanol. However, it soon became clear that the effects of ethanol in the absence of stress were sufficiently well-defined to merit investigation, and the results are presented below.

EXPERIMENTAL

Treatment of animals. Albino rats weighing 140–160 g. were used. Any animal that objected to normal handling was rejected. The general procedure was to administer the ethanol, and after various known times to kill the animal, remove the brain and carry out the chemical determinations.

Ethanol was administered in aqueous solution by stomach tube. The dose used was 3 ml. of 30% (v/v) ethanol/150 g. rat, unless otherwise stated.

The animals were killed by immersion in liquid O_2 for 2–3 min. The head was sawn off and the skull was exposed. By careful saw-cuts the skull could be removed without damaging the brain, which could then be levered out, generally, in one piece. The excision took 5–6 min., and during this time the head and instruments were kept cold by frequent immersion in liquid O_2. The freshly excised frozen brain was weighed and homogenized at 0° immediately.

Blood samples were taken under ether anaesthesia. The blood was collected in citrated syringes from the inferior vena cava, care being taken not to include any air bubbles, and transferred immediately to stoppered tubes each containing 5 ml. of ice-cold 10% (w/v) trichloroacetic acid, well mixed and weighed. If the brain of the same animal was required it was killed by immersion in liquid O_2 immediately after the blood sample was taken, and the brain removed as described above.

In some experiments the aldehyde-dehydrogenase inhibitor tetraethylthiuram disulphide was administered 16 hr. before the ethanol. The inhibitor was given as an aqueous suspension by stomach tube (4 ml./150 g. rat). The suspension was made up by mixing 300 mg. each of

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tetraethylthiuram disulphide and sodium alginate and two drops of Teepol. Water (30 ml.) was added in small portions and the mixture ground in a mortar until a creamy suspension was obtained.

Chemical determinations. Lactate was determined by the method of Barker & Summerson (1941), pyruvate and α-oxoglutarate by that of Koepsell & Sharpe (1952), and acetaldehyde by that of Stotz (1943). Duplicate determinations were made on each brain or blood sample.

Determination of oxidized nicotinamide–adenine dinucleotide in rat brain. The method used for the determination of NAD^+ was based on those of Slater & Sawyer (1962) and Villee (1962, and personal communication). The freshly excised frozen brain was transferred to 5 ml. of ice-cold 6% (v/v) perchloric acid containing nicotinamide at a final concentration of 50 mM (McIlwain, 1955, p. 137) and, after being weighed, homogenized at 0°. The precipitate was separated by centrifuging at 0–4° and the supernatant decanted.The precipitate was washed with 5 ml. of cold 6% (v/v) perchloric acid, and the combined washings and supernatant, partially neutralized with 4 ml. of ice-cold N-NaOH, were made up to 20 ml. with water. The solutions were kept at 0° until required.

Three cuvettes (1 cm. light-path) were prepared containing: (1) distilled water; (2) and (3), 3 ml. of buffered dye solution (2,6-dichlorophenol-indophenol dissolved in 0·1 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 7·23, to give an extinction of 0·9 at 600 mμ), 0·2 ml. of ethanol, 0·1 ml. of brain extract or of standard NAD^+ solution, and 0·05 ml. of *N*-methylphenazonium methosulphate (L. Light and Co. Ltd.) (3 mg./ml. in water).

The contents of cuvettes 2 and 3 were well mixed and allowed to stand in the dark for 5 min. Then 0·05 ml. of alcohol dehydrogenase (ex yeast, L. Light and Co. Ltd.) (6 mg./ml. in water) was added to cuvette 3 and the contents were well mixed; 30 sec. after the addition of the enzyme cuvettes 2 and 3 were read (on a Unicam SP. 500 spectrophotometer) at 600 mμ against water. Subsequently cuvette 3 was read every 30 sec. for a further 3 min. and cuvette 2 at 2 and 3·5 min. after the addition of the enzyme. Under these conditions the rate of dye reduction was linear with time and also a linear function of the coenzyme concentration over the range 0·5–0·25 μm-mole of coenzyme/cuvette.

With each set of determinations a blank (no NAD^+ present) and three NAD^+ (L. Light and Co. Ltd.) standards were run. The standards were made up from a stock solution of NAD^+ containing 1 μmole of NAD^+/ml. in 0·1 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 6·9. This solution was stable for 2 weeks at 4° but it was tested daily as a routine measure (Horecker & Kornberg, 1948).

Duplicate determinations were made on each brain extract and NAD^+ standard solution. The recovery of added NAD^+ was quantitative (97%).

RESULTS

The administration of liquid by stomach tube appeared to be slightly stressful to the rat, and since stress is known to affect the concentrations of certain substances in the brain (Richter & Dawson, 1948; Richter & Crossland, 1949; Dawson & Richter, 1950) it was desirable to ascertain the magnitude and duration of any changes due to the insertion of the stomach tube, so as to separate the effects of the ethanol from those of its mode of administration. Accordingly several rats were each given 3 ml. of water by stomach tube and the brain lactate concentration was determined (Fig. 1). There was a sharp increase in the brain lactate concentration but within 3 min. it had returned to the normal level.

The changes in the blood and brain acetaldehyde concentrations after the administration of ethanol are shown in Fig. 2. The course of events in the brain during the first few minutes after the ingestion of ethanol was rather obscure; there was a suggestion of a very sharp peak in the acetaldehyde concentration (Fig. 2*b*) at about 4 min. and further evidence for the occurrence of some violent but short-lived reaction at about this time is given below. The second and third peaks were progressively lower and longer.

The concentration–time curve for blood acetaldehyde (Fig. 2*a*) was very similar in shape to that for brain acetaldehyde, and within the limits to be expected, in phase with it. The biggest discrepancy occurred in the first 15 min., and the probable cause is that for an appreciable part of this time (5–6 min.) the animals that yielded the blood curve were anaesthetized; the effect became smaller as the time between dosing and taking the sample became longer and by about 25 min. had almost disappeared. A further limit was imposed on the sharpness of the concentration–time curve for blood acetaldehyde by the fact that it was not easy to draw a blood sample exactly at a predetermined time. The values plotted at any given time are therefore the means of the samples drawn within a minute of the time indicated.

The effect of tetraethylthiuram disulphide was to transform the wave-like form of the concentration–

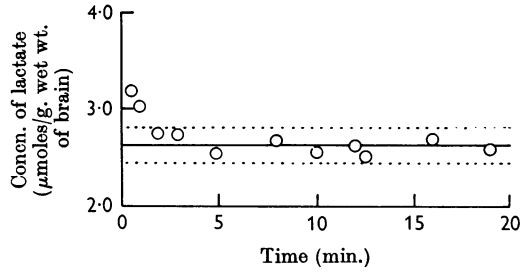

Fig. 1. Effect of administering 3 ml. of water by stomach tube on the lactate concentration of the rat brain. Water was administered at zero time and the animals were killed at various times thereafter. Each point is the mean of determinations on four brains. The horizontal continuous and broken lines represent the mean normal ± S.E.M.

time curves for both blood and brain acetaldehyde into a single peak at 20 min.

The effect of the ingestion of ethanol on the concentrations of some normal metabolites of rat brain was studied (Fig. 3). The normal, undisturbed concentrations of these metabolites in the brain were: lactate, $2{\cdot}62 \pm 0{\cdot}19$ (15); NAD^+, $0{\cdot}201 \pm 0{\cdot}009$ (6); pyruvate, $0{\cdot}112 \pm 0{\cdot}007$ (11) and α-oxoglutarate $0{\cdot}017 \pm 0{\cdot}004$ (11), all expressed as the means ± S.E.M. in terms of μmoles/g. wet wt., with the numbers of determinations in parentheses.

The principal points of interest in Fig. 3 are: (i) the changes in the concentrations of lactate, NAD^+ and α-oxoglutarate (Figs. 3*a*, *b* and *d* respectively) were in phase with the fluctuations of the brain acetaldehyde concentration, but those of the pyruvate concentration (Fig. 3*c*) were 180° out of phase; (ii) the concentration–time curve for brain lactate showed the stressful effect of the stomach tube in the first 2 min.; the high concentration of pyruvate and the low concentration of α-oxoglutarate at 2 min. were also stress effects (unpublished work); (iii) there was a suggestion of a peak in the concentration–time curve for lactate at about 4 min. and a minimum in that of pyruvate at the same time (cf. Fig. 2*b*); (iv) the concentrations of all the normal metabolites returned to their normal, undisturbed levels by about 60 min. while

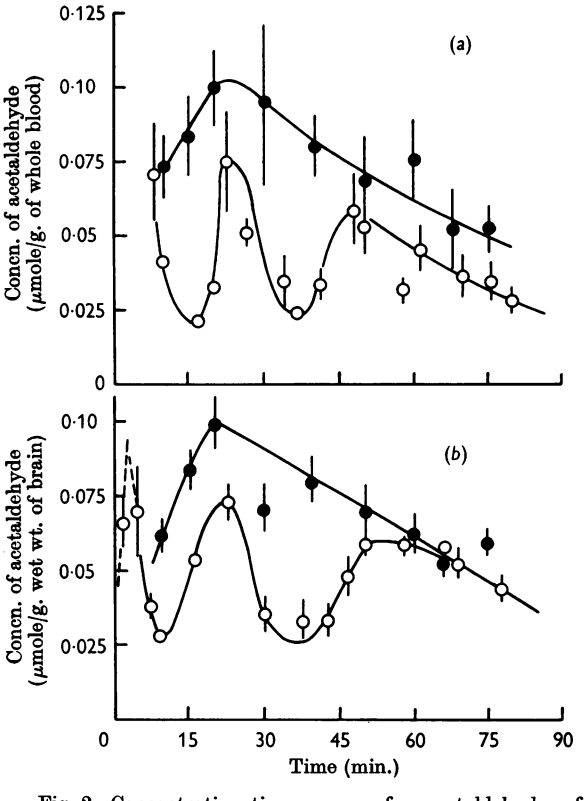

Fig. 2. Concentration–time curves for acetaldehyde of (*a*) blood and (*b*) brain of the rat produced by the ingestion of ethanol [3 ml. of 30% (v/v) ethanol/150 g. rat by stomach tube]. Ethanol was administered at zero time and the animals were killed at various times thereafter. Each point is the mean of determinations on six to twelve brains, the vertical bars representing S.E.M. ○, Effect of ethanol alone; ●, effect of ethanol 16 hr. after pretreatment with tetraethylthiuram disulphide (40 mg./150 g. rat). The time-scale is the same in (*a*) and (*b*).

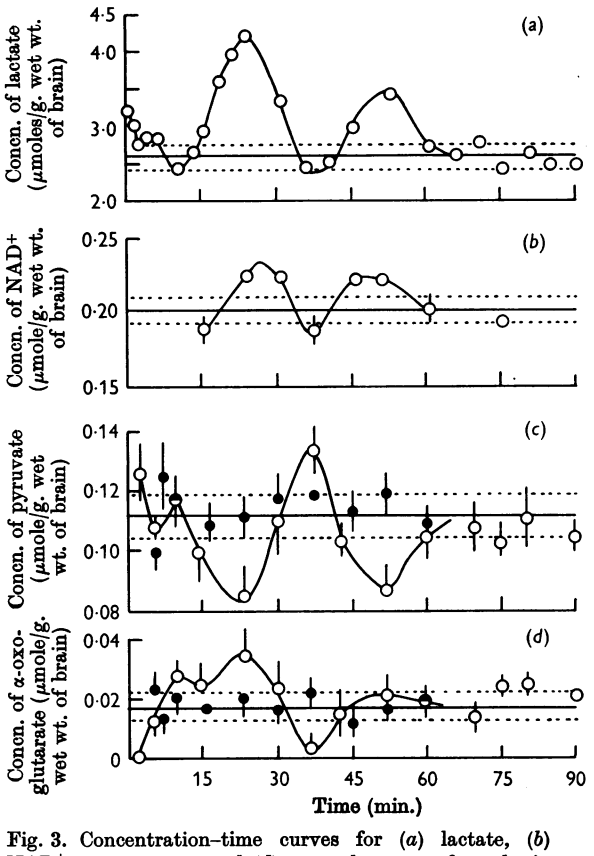

Fig. 3. Concentration–time curves for (*a*) lactate, (*b*) NAD^+, (*c*) pyruvate and (*d*) α-oxoglutarate of rat brain induced by the ingestion of ethanol [3 ml. of 30% (v/v) ethanol/150 g. rat by stomach tube]. Ethanol was administered at zero time and the animals were killed at various times thereafter. The horizontal continuous and broken lines represent the mean normal ± S.E.M. Each point is the mean of determinations on four brains for lactate and six to twelve brains in all other cases, the vertical bars representing ± S.E.M.; for lactate S.E.M. values were not calculated and no vertical bars are plotted, but in all other cases where no vertical bar appears it is contained within the symbol; where overlapping might occur the bar is 1 S.E.M. long, measured from the centre of the symbol. ○, Effect of ethanol alone; ●, effect of ethanol 16 hr. after pretreatment with tetraethylthiuram disulphide (40 mg./150 g. rat). The time-scale is the same in (*a*), (*b*), (*c*) and (*d*).

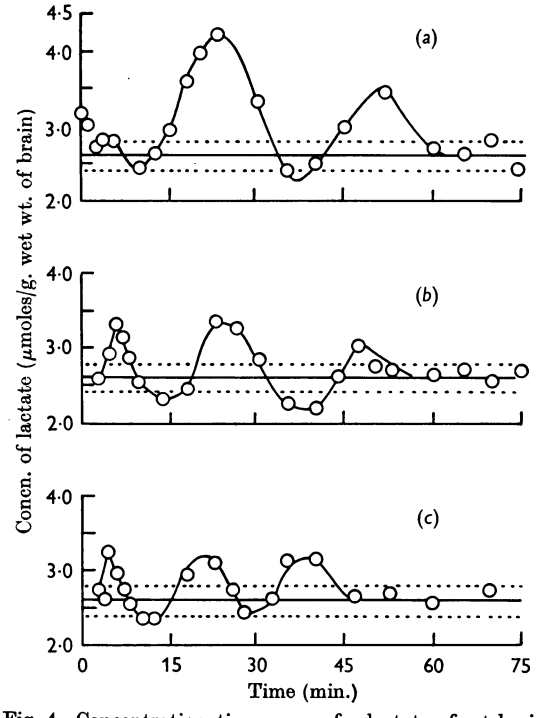

Fig. 4. Concentration–time curves for lactate of rat brain induced by different doses of ethanol: (*a*) 3 ml. of 30% (v/v) ethanol/150 g. rat, i.e. 4·73 g. of ethanol/kg. rat; (*b*) 3 ml. of 20% (v/v) ethanol/150 g. rat, i.e. 3·16 g./kg.; (*c*) 3 ml. of 10% (v/v) ethanol/150 g. rat, i.e. 1·58 g./kg. Ethanol was administered by stomach tube at zero time and the animals were killed at various times thereafter. Each point is the mean of determinations on four brains. The horizontal continuous and broken lines represent the mean normal ± S.E.M. The time-scale is the same in (*a*), (*b*) and (*c*).

at the same time there were appreciable amounts of acetaldehyde in both blood and brain; (v) the concentrations of pyruvate and α-oxoglutarate remained within the normal range after pretreatment with tetraethylthiuram disulphide.

Fig. 4 shows the effect of different doses of ethanol on the concentration of lactate in the brain. Over a fairly wide range of ethanol dosages the concentration–time curves for brain lactate were of the same general form; the only difference was that at the lower doses the maxima were lower and the 'period' of the oscillations was shorter. The most striking feature of the curves was the presence in Figs. 4 (*b*) and (*c*) of a sharp peak at about 5 min., of which there was only a suggestion in Fig. 4 (*a*).

DISCUSSION

Acetaldehyde is present in significant quantities in mammalian tissues only after the ingestion of ethanol and is the product of the first step in the oxidation of the alcohol. Compared with ethanol, acetaldehyde is highly toxic (Westerfeld, 1955) and is rapidly oxidized *in vivo* (Westerfeld, McKibbin, Roemmelt & Hilfinger, 1949) by way of acetyl-CoA and the tricarboxylic acid cycle (Chou & Lipmann, 1952).

The liver is the principal site of ethanol and acetaldehyde metabolism (Lubin & Westerfeld, 1945), but despite its action on the central nervous system there is little evidence that the brain is capable of metabolizing ethanol. Dewan's (1943) claim to have prepared from brain a system capable of oxidizing ethanol has not been confirmed (Beer & Quastel, 1958), and ^{14}C-labelled ethanol incubated with respiring brain tissue failed to yield ^{14}C-labelled carbon dioxide, fatty acids or cholesterol (Bartlett & Barnet, 1949; Masoro, Abramovitch & Birchard, 1953; Westerfeld & Schulman, 1959). This conflict of evidence led to the suggestion that the effects of ethanol were due to its conversion into acetaldehyde. However, investigation revealed a lack of correlation between ethanolic intoxication and the pharmacological effects of acetaldehyde *in vivo* (Westerfeld, 1955; MacLeod, 1950). The present results show that during ethanol catabolism there was some disturbance of brain metabolism; but they throw no light on the interaction, if any, between the central nervous system and ethanol. They do, however, show how the brain reacts to the presence of acetaldehyde.

Acetaldehyde in the blood, after a single dose of ethanol, decays exponentially (Westerfeld, 1955). But Fig. 2 (*a*) shows that for the first 50 min. after the ingestion of ethanol the blood acetaldehyde concentration underwent a series of fluctuations, and only after 50 min. was there a steady decay. These two points of view are not necessarily in conflict, for the bulk of previous work on acetaldehyde metabolism was not concerned with events during the first hour after the administration of ethanol. The causes of the fluctuations in the blood acetaldehyde concentration were not investigated; they will probably be found in the liver.

The similarity of the concentration–time curves for brain acetaldehyde to the corresponding curves for blood acetaldehyde is attributable to the low molecular weight of acetaldehyde and its high solubility in both water and fat solvents. Few, if any, animal tissues would present a serious barrier to its rapid diffusion; it readily passes into the brain (MacLeod, 1950) and there can be little doubt that the brain acetaldehyde concentration was determined principally by the blood acetaldehyde concentration. Although acetaldehyde is metabolized by the brain (McIlwain, 1955, p. 230) the quantity so metabolized is evidently small, for the concentration in the brain did not differ

markedly from that in whole blood (Fig. 2). Such metabolism implies the existence in brain of an enzyme system capable of converting acetaldehyde, presumably, into acetate (Lundquist, Tygstrup, Winkler, Mellemgaard & Munck-Petersen, 1962). The results (Fig. 3) indicate that this oxidation impinged on the carbohydrate metabolism of brain sufficiently to cause very marked fluctuations in the steady-state concentrations of at least three intermediates and one coenzyme.

The central problem of these events in the brain is the link between acetaldehyde oxidation and carbohydrate metabolism. After pretreatment with tetraethylthiuram disulphide (Antabuse, a compound used in the treatment of alcoholism) the fluctuations in pyruvate and α-oxoglutarate concentrations were abolished and the link between acetaldehyde oxidation and carbohydrate metabolism in the brain was broken (Figs. 3*c* and *d*). Tetraethylthiuram disulphide inhibits acetaldehyde metabolism by competing with NAD^+ for the active centres of aldehyde dehydrogenase (Graham, 1951). Since NADH is required for the enzymic reduction of pyruvate, NAD is capable of linking acetaldehyde dehydrogenase and lactate dehydrogenase.

Accordingly the oxidation of one molecule of acetaldehyde is linked to the reduction of one molecule of pyruvate, but it is unlikely that such simple stoicheiometrical relationships would be demonstrable *in vivo*. The coenzyme-linked system *in vivo* is a small part of a complex network of which most of the reactions are reversible and operating under steady-state conditions. Thus the net effect of any disturbance is not necessarily directly proportional to its magnitude. The most that can be said is that if the metabolism of pyruvate is otherwise unchanged the oxidation of acetaldehyde might be accompanied by a decrease in the steady-state concentration of pyruvate and an increase in that of lactate; and, up to a point, the higher the acetaldehyde concentration, the higher its rate of oxidation and the more the steady-state concentrations of pyruvate and lactate would diverge from the normal. These conclusions are supported by the results if the period from 12 to 60 min. after the ingestion of ethanol is considered. After 60 min. a relatively high acetaldehyde concentration accompanied normal concentrations of other metabolites; perhaps this acetaldehyde concentration can be counteracted by metabolic homoeostasis, or perhaps the absence of rapid fluctuations in the blood acetaldehyde concentration allowed homoeostasis to be effective.

The most violent disturbance took place very soon after the ethanol was administered. This was reflected objectively in the sharp peaks in the brain acetaldehyde concentration (Fig. 2*b*) and in the lactate concentration (Fig. 4) at about 3–5 min. It was also repeatedly noticed that at precisely this time after the administration of the ethanol the rats' teeth chattered for about 30 sec.; this was the only consistent behavioural effect observed. It was probably a stressful experience, and for this reason it is not profitable to try to explain the observed changes in the first 10–12 min. in terms of acetaldehyde metabolism alone.

The disturbance in carbohydrate metabolism was not confined to lactate and pyruvate: α-oxoglutarate was also affected (Fig. 3*d*). The curve may be divided into the same three parts of which the first and third do not call for further comment. The second (i.e. 12–60 min.) contained fluctuations the maxima and minima of which coincided respectively with high and low rates of acetaldehyde oxidation and were probably connected with the oxidation of the resulting acetate.

SUMMARY

1. The administration of ethanol to the normal rat was followed by a series of wave-like fluctuations in the acetaldehyde concentrations of blood and brain.

2. These fluctuations lasted for about an hour and were followed by a steady decline in the acetaldehyde concentrations of both blood and brain.

3. Fluctuations in the lactate, pyruvate, α-oxoglutarate and NAD^+ concentrations of brain also occurred at the same time. All returned to their normal concentrations in the steadily declining phase of the concentration–time curve for acetaldehyde.

4. The time and phase relationship of all these oscillations and the observation that they could be eliminated by an aldehyde-dehydrogenase inhibitor suggested: (*a*) that the rat brain contains an aldehyde dehydrogenase; (*b*) that the oxidation of acetaldehyde by the rat brain *in vivo* is linked through NAD to pyruvate reduction.

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The Reaction of Glutathione with Serum Albumin, Gluten and Flour Proteins

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(*Received 5 November* 1962)

Previous work (Hird, 1962) has shown that the rate of reaction of GSH with the disulphide bonds of native serum albumin and insulin to form GSSG was slow, but that after digestion with trypsin or chymotrypsin or both the reaction was rapid. The formation of GSSG in such systems results from two thiol–disulphide-exchange reactions (1 and 2).

$$GSH + Prot \cdot S \cdot S \cdot Prot \rightleftharpoons GSS \cdot Prot + Prot \cdot SH \quad (1)$$

$$GSS \cdot Prot + GSH \rightleftharpoons GSSG + Prot \cdot SH \quad (2)$$

It is therefore possible that, with the native proteins, the first reaction leading to the formation of the mixed disulphide between GSH and the protein could occur and not be detected by the method used, namely oxidation of $NADPH_2$ by glutathione reductase. This assay method requires optical clarity and cannot be used in opaque systems; its application is also restricted to conditions that do not destroy enzymic activity. To overcome these difficulties, a polarographic method has been used to follow the disappearance of GSH in the systems studied.

In addition to the study of the reaction of GSH with serum albumin the method has been applied to a related study of the flour–water system in dough. It has been established that the removal of thiol groups markedly toughens dough and increases its viscosity (Goldstein, 1957; Bloksma, 1958; Frater, Hird, Moss & Yates, 1960; Mecham, 1959; Bloksma & Hlynka, 1960); the addition of a small amount of a thiol has the opposite effects (Frater *et al.* 1960). From these collected observations it has been concluded that thiol–disulphide-exchange reactions substantially determine the rheological properties of dough. The experimental facts, however, relate only to the removal and addition of thiol groups and not to participation of disulphide bonds as such. Accordingly we have attempted to obtain more experimental information on the interaction of GSH and GSSG with the disulphide bonds and thiol groups respectively of proteins in dough.

MATERIALS AND METHODS

Materials. Serum albumin (bovine) was purchased from the Commonwealth Serum Laboratories and had been crystallized 15 times. Trypsin and chymotrypsin were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and GSH fromHo fmann–La Roche and Co. Ltd., Basle, Switzerland. Methylmercuric iodide was prepared by the method of Maynard (1932).

Gluten was a gift from Barret Bros., Melbourne, Australia, and had been prepared by the extraction of flour with acetic acid. Reduced gluten was prepared by the reduction of a gluten suspension (100 g.) in 10 l. of aq. 0·1 M-sodium borohydride. After reduction for 1 hr. at 50° the suspension was acidified to pH 6·5 to decompose the excess of sodium borohydride. The product, which is insoluble at pH 6·5, was collected by centrifuging and washed four times with water (1 l.), and the residue was freeze-dried. Before reduction the gluten contained 11·5 moles of disulphide groups/10^5 g. and no thiol groups; after reduc-