5. Tobacco mosaic virus, *Bacillus megaterium* and cells of the Ehrlich ascites tumour have been treated with ³⁵S-labelled mustard gas; the extent of reaction with nucleic acid has been determined and shown to be about the same as that with protein. The site of reaction with nucleic acid has been shown, as in the case of pure nucleic acid, to be at N-7 of guanine moieties.

6. Mice bearing the Ehrlich ascites tumour have been injected with ³⁵S-labelled mustard gas and the nucleic acid of the tumour cells shown to have reacted in the same way as *in vitro*.

7. Previous work on the reactions of alkylating agents with nucleic acids is discussed in relation to the present findings.

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The Simple Monosubstituted Guanidines of Mammalian Brain

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Although observations have been made on individual monosubstituted guanidine derivatives of the brain, there appears to have been no systematic investigation of their total amount or number. Such a study has now been performed. Values for the concentration of total monosubstituted (Sakaguchi-positive) guanidines have been obtained, in

guinea-pig, ox and rat brain; paper-chromatographic studies have confirmed the presence of arginine, glycoylamine (guanidoacetic acid) and γ -guanidobutyric acid, and have indicated the occurrence of taurocyamine (guanidoethane sulphonic acid). Approximate values for concentrations of the individual bases have also been obtained.

METHODS

Materials. Commercial arginine and glycocyamine were recrystallized as the hydrochlorides; commercial creatine was purified with charcoal and recrystallized from water (King, 1930). γ -Guanidobutyric acid was prepared from cyanamide (Engeland & Kutscher, 1910) and recrystallized as the hydrochloride (m.p. 184°, corr.). Taurocyamine was prepared from isothioureia (Schütte, 1943; Morgue, 1948; Van Thoai & Robin, 1954); recrystallization of the free base from water, ethanol or ethanol-acetone yielded white crystals, melting sharply at 267° uncorr. (Engel, 1875; Morrison, Ennor & Griffiths, 1958). Both synthetic products chromatographed identically, in six solvents, with samples of the authentic compounds kindly given by Dr I. Smith.

Commercial 8-hydroxyquinoline was recrystallized from ethanol (m.p. 72–73°, corr.). Chromatographic solvents were of chromatography grade where available or else analytically pure. Other reagents were analytic grade unless specified otherwise.

Rats and guinea pigs were obtained commercially; no attempt was made to control strain or sex differences. Ox brain was obtained fresh at a slaughter house.

Paper chromatography. Descending chromatograms were developed at room temperature. Standard solvents were butan-1-ol-acetic acid-water (12:3:5, by vol.) and butan-1-ol-pyridine-water (1:1:1, by vol.); in two-dimensional chromatography the acidic solvent was used first (Smith, 1958). Other solvents were: propan-2-ol-NH₃ soln. (sp.gr. 0.88)-water (73:4:23, by vol.) (Robin, 1954); propan-2-ol-acetic acid-water (15:1:4, by vol.) and butan-1-ol-formic acid-water (77:11:12, by vol.). On Whatman no. 1 chromatography paper, unwashed, up to 2 mg. could be applied on a single spot. On Whatman no. 3MM paper, used for larger quantities, the bases ran more rapidly and diffusely. Compounds were detected by spraying with Sakaguchi reagents, which clearly show 2 μ g. of monosubstituted guanidines and give no colour even with 2.5 mg. of creatine (Smith, 1958). For creatine, pentacyanoferrate spray was used (Smith, 1958). R_F values in the first three solvents were as described by Smith (1958) and Robin (1954); values in the other two are summarized in Table 1. Although the temperature was not controlled, variations in R_F tended to be small (s.d. about 10%). In particular experiments values up to $\pm 25\%$ from the average occurred with pure compounds and impure extracts; however, controls, run on all chromatograms, retained their relative positions as discrete spots. Recoveries of the bases on elution with N-HCl were 80–100% (four determinations each on arginine, glycocyamine and γ -guanidobutyric acid).

Assay

Monosubstituted guanidines were estimated by the modification of the Sakaguchi reaction of Dubnoff (1941, 1957), in which up to 0.2 mg. of creatine or creatinine gave an insignificant colour; greater quantities gave irregular but significant readings.

With precise control, and rapid, reproducible addition of hypobromite the reaction is quantitative in the range 0.02–0.15 μ mole. In 100 determinations in various extracts over the course of a year, the average difference between duplicates was $4 \pm 4\%$ (s.d.); recoveries from tissue extracts were quantitative (see below). The bases have different extinc-

tion coefficients (Table 2). Arginine, known to be present in relatively large amounts (see Discussion), was chosen as colorimetric standard and values are expressed as μ moles of arginine.

Tissue extracts

Removal of tissue. Usually rats or guinea pigs were killed by a blow on the neck, the brain was removed, blotted on Whatman no. 50 paper and either ground immediately in deproteinizing agent in a tared, all-glass homogenizer or rapidly weighed and then fixed. Time from blow to fixing was no more than 3.5 min.; no difference caused by the two methods of obtaining weight was noted. Usually cerebral hemispheres and cerebellum were used; when comparison with frozen material was needed, only cerebral hemispheres were taken.

In one series of experiments, young rats (2 weeks, 100 g.) were dropped head-first into liquid nitrogen and the cerebral hemispheres were chiselled out (Richter & Dawson, 1948; McIlwain, Buchel & Cheshire, 1951). Samples of tissue allowed to come to room temperature contained, on examination, no bone.

Ox brain, removed at the slaughter house, was transported to the laboratory at ice-bath temperature.

Deproteinization. Unless described otherwise, homogenates were prepared at ice-bath temperature and centrifuged for 20 min. at 10 000 g and $0^\circ \pm 2^\circ$. In calculating tissue concentrations from portions of the supernatant, the tissue was assumed to contribute a volume of water equal to 80% of its wet weight to the homogenate.

Table 1. R_F values of guanidine bases

Chromatograms were developed at room temperature, on Whatman no. 1 chromatography paper, and spots were detected with either Sakaguchi or pentacyanoferrate spray reagents (see text). Figures represent the means of at least four determinations; s.d. is about 10%.

Base	R_F	
	Solvent: butanol-formic acid-water (77:11:12 by vol.)	Solvent: propan-2-ol-acetic acid-water (15:1:4 by vol.)
Arginine	0.07	0.17
γ -Guanidobutyric acid	0.45	0.56
Glycocyamine	0.23	0.38
Taurocyamine	0.11	0.29
Creatine	0.35	0.33

Table 2. Extinction coefficients of guanidine bases in Sakaguchi reaction

Method of Dubnoff was used (see text). The value of the extinction coefficient is followed by the s.d. and the number of duplicate determinations. The μ molar extinction coefficient is given.

Base	$10^4 \epsilon$
Arginine hydrochloride	4.6 ± 0.6 (28)
γ -Guanidobutyric acid hydrochloride	6.4 ± 0.6 (8)
Glycocyamine hydrochloride	5.5 ± 0.6 (6)
Taurocyamine (free base)	9.5 ± 0.1 (4)

Extracts with trichloroacetic acid (10%, w/v) were prepared by grinding brain (0.2–0.4 g./ml.) in the acid and centrifuging. Assay of the clear to slightly opalescent supernatant showed no dilution effects: there was no systematic variation with different sized portions or different concentrations of tissue in deproteinizing agent.

Extracts with tungstic acid were prepared by grinding the tissue (0.2–0.4 g./ml.) in fresh, dilute, phosphate-stabilized tungstic acid reagent (Caraway, 1958) and centrifuging. Since material interfering with the Sakaguchi assay was extracted by tungstic acid (Van Pilsum, Martin, Kito & Hess, 1957), two different-sized samples of the supernatant were taken (e.g. 0.5 and 1.0 ml.) and the results were extrapolated to zero-tissue concentration (e.g. the difference between concentration calculated from the 0.5 ml. and from the 1.0 ml. samples was added to the concentration calculated from the 0.5 ml. sample).

In preparing metaphosphoric acid extracts, all solutions of the reagent must be kept cold (0°–5°). A stick of metaphosphoric acid (NaPO₃-stabilized) was thoroughly washed, covered with enough water to make a 20% solution and left overnight. This solution was shaken and diluted to 6% (w/v); brain (up to 0.2 g./ml.) was ground in dilute reagent and centrifuged. The supernatant was clear but foamed on shaking.

The following procedure, performed as rapidly as possible, was adopted for deproteinization by heating (Hunter, 1957). A homogenizer and pestle containing 20 ml. of water were placed in a boiling-water bath (95°–99°). Brain (up to 4 g.) was weighed to the nearest 0.1 g., the homogenizer was removed from the bath, 0.3 m-equiv. of acetic acid/g. of brain was added to the water, the tissue was homogenized in the hot dilute acid and the homogenate (pH about 5.8) was returned to the boiling-water bath for 10 min. Material tended to clot at the top and bottom of the suspension. Centrifuging gave a clear to slightly opalescent supernatant which could, with care, be removed by Pasteur pipette from the very soft precipitate. Ox brain was deproteinized by blending the tissue (top-drive macerator) in 3–5 vol. of hot dilute acetic acid (80°–90°, 3 m-equiv. of acetic acid/g. of tissue), and boiling the suspension for 10 min. and centrifuging for 15 min. at 1400 g. at room temperature. Supernatants were cloudy and foamy.

Purification of extracts for paper chromatography

Initial observations of the presence of guanidine derivatives in cerebral extracts were made by B. M. Bollard & H. McIlwain (unpublished work) during a study of cerebral purines.

Purification on ion-exchange resin. The alkaline constituents of tissue extracts prepared with tungstic acid from about 5 g. of brain were concentrated on Amberlite IR-120(H) (100–200 mesh), which was prepared by alternately washing with 5N-HCl (three times) and with 2N-NaOH (twice) and stored dry in the H⁺ form. Eluents with NH₃ soln. of thoroughly washed resin gave no Sakaguchi reaction on paper but reacted irregularly in the assay, corresponding to 0.3–3.0 µg. of arginine/ml. of eluent.

The 60–70% of the tungstic acid supernatant which could be removed (by Pasteur pipette) from the precipitate was added to a column of resin (2.5 g./g. of tissue; height: diameter > 9:1). The column, run at 0.5 ml./min., was washed with water (20 ml./g. of tissue) and with 2N-NH₃ soln. (25 ml./g. of tissue). The water eluate was discarded

and the ammonia eluate was vacuum-distilled to dryness and taken up in two 2 ml. portions of N-HCl; a white insoluble residue was removed by gravity filtration (Whatman no. 43 paper), and the filtrate evaporated to dryness on the rotary evaporator (Aves, 1959); the residue was dissolved in 2 ml. of N-HCl/mg. of guanidine. Quantitative estimates of recovery are prevented by the variable resin blank; assay suggested recovery from the column of 80–90%, recovery from the tissue of 50% and a 50-fold concentration of monosubstituted guanidines (to 2% of non-protein dry weight).

In four control experiments, creatine (1.5 mg./g. of resin) was taken through the procedure; chromatograms showed no material reacting with the Sakaguchi reagents.

Large-scale preparations. Extracts of about 0.5 kg. of ox brain which had been deproteinized by heating were freeze-dried, the dry residue was taken up in water (300 ml./kg. of tissue), diluted 2 in 5 with tungstic acid reagent and centrifuged. The foamy yellow extract was purified on Amberlite IR-120(H), the conditions being similar to those for small quantities of tissue; dimensions and rate of the column were adjusted to the larger samples. The HCl-insoluble residue, removed by gravity filtration, contained approx. 20%, and the final extract for chromatography approx. 50%, of the material reacting with the Sakaguchi reagent added to the resin. These extracts were further purified by paper chromatography. The extract was streaked (50 µl./cm.) across Whatman no. 3MM paper, developed in butanol-acetic acid-water, and the bases were eluted; positions were shown by markers of known bases (see Results section), since the extract concentration was too low for clear spots. The eluates were concentrated either by freeze-drying or on the film evaporator.

RESULTS

Concentration of total monosubstituted guanidines

Concentration in different extracts. Table 3 summarizes the results of Sakaguchi assay on various extracts of adult-rat brain. Trichloroacetic acid and tungstic acid extracts have essentially the same concentration (*P* value of the difference > 0.01). Both methods are reproducible and give good recoveries: in trichloroacetic acid, arginine recovery was 99 ± 4% (S.D.; four determinations) and

Table 3. *Concentration of monosubstituted guanidines in rat-brain extracts*

The cerebrum was removed at room temperature and ground in deproteinizing agent within 3 min. of death (see text). Assay was by the method of Dubnoff. Values are expressed as µmoles of arginine/g. wet wt. (assuming 80% of wet wt. is water), followed by mean deviation and the number of animals examined. Duplicate determinations were made on extracts from each animal.

Deproteinizing agent	Concentration (µmole/g. of fresh tissue)
Trichloroacetic acid	0.21 ± 0.03 (4)
Tungstic acid	0.18 ± 0.02 (4)
Metaphosphoric acid	0.33 ± 0.02 (2)
Somogyi reagents	0.11 ± 0.02 (2)
Heat	0.7 ± 0.1 (4)

γ -guanidobutyric acid recovery was $95 \pm 7\%$ (S.D.; four determinations): in tungstic acid, γ -guanidobutyric acid recovery was $101 \pm 6\%$ (S.D.; four determinations). Metaphosphoric acid extracts give a significantly higher level, possibly because they contain proteins or peptides which give a Sakaguchi reaction.

Extracts which were deproteinized by heating showed a concentration of guanidine three to five times that with trichloroacetic acid. The increase could be caused by breakdown of a chemical precursor, by release of free arginine from proteins, or by proteins or peptides left in solution under these conditions. Chemical breakdown could not give rise to significant amounts of non-endogenous monosubstituted guanidines; all bases identified were also found in tungstic acid extracts. Even the active brain proteinases should be rapidly inactivated at 95° (Richter, 1959; McIlwain, 1959). The following evidence supports the third interpretation: (1) The extracts contain much surface-active material. (2) Although biuret assay (Layne, 1957) was negative, implying the presence of less than 2.5 mg. of protein/g. of tissue in the solution, Folin-Ciocalteu assay for aromatic amino acids (Layne, 1957) showed 3.9 mg. of protein/g. of tissue left in solution. (3) Tungstic acid precipitated all (about 0.3μ -mole of guanidine/g. of tissue; approx. 2 mg. of protein/g. of tissue), and trichloroacetic acid precipitated half of the excess of material reacting with the Sakaguchi reagents.

Concentrations in different tissues. Table 4 summarizes the results of Sakaguchi assay on trichloroacetic acid extracts of brains derived from different sources. No difference was noted, in adult rats and guinea pigs, between extracts of cerebral hemispheres and those of combined cerebral hemispheres and cerebellum. Adult rat and adult guinea-pig

brains contain essentially the same concentration of monosubstituted guanidines ($P > 0.01$). The concentration in ox brain is higher and more variable (cf. standard deviations): the differences could arise from proteolysis or similar breakdown during the rather more drastic pretreatment of the ox tissue (see Methods section). The difference in concentration between young rat brain frozen *in situ* and that obtained normally, and between young and adult rats, are both barely significant ($0.01 < P < 0.001$). In these studies, only one batch of young (newly weaned) animals was used.

Identification of monosubstituted guanidines

Paper chromatography of the alkaline constituents, concentrated by column chromatography from rat brains, in butanol-acetic acid-water (three experiments, with six animals each), and elution and rechromatography in butanol-pyridine-water (one experiment), both revealed four materials, which moved with arginine, glycoeyamine, taurocyamine and γ -guanidobutyric acid respectively. The extracts were too impure to give good two-dimensional chromatograms; even in one-dimensional chromatography, it was necessary to add approx. 2 mg. of total material to the paper to obtain spots bright enough and small enough (about 1.5 cm. diam.) to give a clear colour above background (see Smith, 1958). From the minimum quantity needed to reveal a particular base (see Methods section), the concentrations of the individual bases in the tissue can be roughly calculated, in μ mole/g., as: arginine, 0.1; γ -guanidobutyric acid, 0.05; glycoeyamine, 0.03; taurocyamine, 0.03.

With extracts of ox brain, two-dimensional chromatography (0.1–0.15 mg.) showed four spots, moving with the known bases (two experiments, each on two ox brains). Eluates obtained from Whatman no 3 MM paper (see Methods section) showed materials which moved with the known bases in the five solvents (see Methods section).

The same four bases were found in ox-brain preparations deproteinized by heating, and purified by solvent extraction and paper chromatography; the procedure was relatively unreplicable. Preparations by other methods, including alkaline silver precipitation (B. M. Bollard & H. McIlwain, unpublished work), electrolytic desalting and Somogyi (1945) precipitating gave no evidence for any other naturally occurring monosubstituted guanidines.

DISCUSSION

Presence of monosubstituted guanidines

Total concentration. The concentration of the monosubstituted guanidines occurring in brain, 0.2 – 0.3μ mole/g., is markedly smaller than the

Table 4. *Concentrations of monosubstituted guanidines in brains from various sources*

Extraction was with trichloroacetic acid; assay was by the method of Dubnoff (see text). Concentrations are expressed as μ mole of arginine/g. wet wt. of tissue (assuming 80% of wet wt. is water); the values are followed by S.D. and the numbers of animals examined. Duplicate determinations were made on samples from each animal.

Tissue	Source	Concentration (μ mole/g. of fresh tissue)
Cerebral hemispheres or combined cere- bral hemispheres and cerebellum	Adult guinea pig	0.24 ± 0.04 (11)
	Adult rat	0.21 ± 0.04 (13)
Cerebral hemispheres	Ox	0.30 ± 0.10 (4)
Cerebral hemispheres	Young rat*	0.29 ± 0.05 (11)
Cerebral hemispheres, frozen <i>in situ</i>	Young rat*	0.21 ± 0.04 (7)

* 2-week-old newly weaned animals, weighing about 100 g.

8–10 μ moles/g. of total creatine (disubstituted guanidines) (Olsen & Klein, 1947). Most of these investigations have been carried out with tissue removed from animals at room temperature, but it has been shown that tissues frozen *in situ* do contain monosubstituted guanidines.

Arginine. Of the individual guanidines, arginine constitutes nearly half of the total. The present value of 0.1 μ mole/g. of rat brain is similar to the 0.12 μ mole/g. of rat brain of 'microbiologically available' arginine (Schurr, Thompson, Henderson, Williams & Elvehjem, 1950) and to the 0.08 μ mole of free arginine/g. of rat brain (Tallan, Moore & Stein, 1954; Clouet, Gaitonde & Richter, 1957). Paper-chromatographic identification of arginine as one of the free amino acids of brain was reported by Irreverre & Evans (1959) and by Clouet *et al.* (1957), but not by Walker (1952) nor by Awapara, Landau & Fuerst (1957).

γ -Guanidobutyric acid. This was reported in cattle brain (Irreverre, Evans, Hayden & Silber, 1957) and, while these investigations were in progress, was isolated from calf brain by Irreverre & Evans (1959) who found 0.03 μ mole/g. of calf brain. We found approximately 0.05 μ mole/g. of rat brain.

Glycocytamine. This was identified as a cerebral constituent by Borsook & Dubnoff (1941) and by Clouet *et al.* (1957). Our value for rat brain (0.03 μ mole/g.) is significantly lower than that reported by Borsook & Dubnoff (0.15–0.3 μ mole/g.) and by Clouet *et al.* (0.1 μ mole/g.). The earlier values are based on column purifications which may not have distinguished glycocytamine from γ -guanidobutyric acid and taurocytamine.

Taurocytamine. This has been identified in brain tissue in the present study. It is a constituent of Crustacea (Van Thoai & Robin, 1954) and mammalian urine (Van Thoai, Roche & Olumucki, 1954).

Other monosubstituted guanidines. In extracts prepared by several radically different procedures, only the four bases discussed above were identified. The existence of another simple, monosubstituted guanidine in brain, in concentrations greater than 0.03 μ mole/g., seems unlikely.

Function of the guanidines

All of the monosubstituted guanidines identified are ω -guanido acids, although the isolation procedures would not be expected to select these amphoteric guanidines. ω -Guanido acids have been shown to have several effects on neural preparations. They act on the inhibitory synapses in isolated crustacean nerve (Edwards & Kuffler, 1959), and they are among the most active of a series of compounds affecting electric activity of the cerebral cortex (Purpura, Girado, Smith, Callan & Grundfest, 1959). Monosubstituted guanidines inhibit the

Pasteur effect in brain slices (Dickens, 1939; McIlwain, 1950). After intracisternal injection of radioactive amino acids into rabbits, activity has been found in Krebs urea-cycle intermediates in the brain (Sporn, Dingman, Defalco & Davies, 1959*a, b*; Sporn, Dingman & Defalco, 1959). Rat-brain homogenates contain a transaminidase, synthesizing guanidines from analogous amines (Pisano & Udenfriend, 1958). Preliminary experiments in this laboratory have suggested the presence of an acid-labile, bound form of guanidines in cerebral tissue (H. McIlwain & J. P. Blass, unpublished observations).

SUMMARY

1. Methods of extracting the monosubstituted (Sakaguchi-positive) guanidines of brain, and of their chromatographic characterization, are described.

2. The occurrence of free arginine, glycocytamine and γ -guanidobutyric acid is confirmed, and evidence for the presence of taurocytamine is presented.

3. Concentrations of these compounds are approximately 0.03–0.1 μ mole/g. of tissue, which corresponds to about 1 % of the free creatine of the brain.

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The Incorporation of Radioactive Amino Acids into Proteins of the Microsome Fraction of Guinea-Pig Liver at very Short Time Intervals after Administration

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The question whether the process of incorporation of labelled amino acids into trichloroacetic acid-precipitable protein, which takes place in cell-free systems, represents part or all of the process resulting in the synthesis of proteins in the corresponding intact cell is an important problem in the interpretation of the results of many investigations into the mechanism of protein biosynthesis. Simkin & Work (1957*a, b*) attempted to obtain information on this question with respect to the microsome fraction of guinea-pig liver. In that work the microsome fraction was labelled with [¹⁴C]amino acids by allowing incorporation to take place either in the intact guinea pig or in a cell-free system of the type described by Zamecnik & Keller (1954) which contains microsome and cell-sap fractions. The microsome fraction was then isolated, and separated into a series of distinct subfractions by a procedure involving successive extraction with solutions of varied ionic strength and pH. The radioactivity of the protein of the subfractions was determined. When the results of labelling under the

two sets of conditions were compared, the pattern of labelling found in the cell-free system appeared to be different from that resulting from incorporation in the intact cell. The difference in behaviour was particularly noticeable in two ribonucleic acid-rich subfractions, *B* and *C*. The reasons for this difference in the labelling pattern were not clear. The shortest period of exposure to radioactive amino acids *in vivo* in this work was 2 min., and, as Simkin (1959) has pointed out, the possibility could not therefore be excluded that the pattern of labelling found in the cell-free system resembled that occurring in the intact liver cell as a result of periods of incorporation less than this. The results of analogous investigations carried out by Sachs (1958) lent support to this view. In order to resolve this question, the labelling of the microsome subfractions of guinea-pig liver occurring *in vivo* has been re-examined, very brief periods of exposure to [¹⁴C]amino acids now being employed. The results obtained support the suggestion made above: there is a similarity in the pattern of labelling occurring in