# PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **286th Meeting** *of the Biochemical Society was held in the Physiology Institute, Newport Road, Cardiff, on Friday, 19 May 1950, when the following papers were read:*

COMMUNICATIONS

The Orientation of Glucuronic Acid Conjugation in Resacetophenone. By K. S. Dodgson. (*Physiology Institute, Newport Road, Cardiff*)

Dodgson & Williams (1949) and Dodgson, Smith & Williams (1950) have shown that the dihydroxybenzene derivatives, 4-chlorocatechol, 4-chlororesorcinol and chloroquinol, form, in rabbits, monoglucuronides which may be formulated as (I), (II) and (III) respectively.


(I)   (II)   (III)
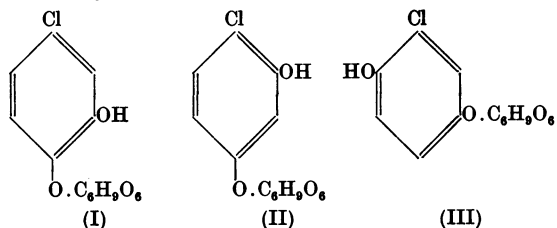
In all these compounds, conjugation with glucuronic acid occurs on the hydroxyl group which is farthest away from the chlorine atom.

It has now been shown that resacetophenone (IV) behaves in similar fashion.

Resacetophenone glucuronide has been isolated previously from the urine of dogs and rabbits by Nencki (1894), who described it as a monohydrated monoglucuronide which melted at approximately 170°.

In the present experiment, this glucuronide was isolated in excellent yield (corresponding to 35 % of the dose) from the urine of rabbits receiving resacetophenone orally. The glucuronide was a monohydrate, m.p. 175–176° (with decomposition) and $[\alpha]_D^{22°} - 99°$ (0·714 % in water). It gave a deep red-purple colour with $FeCl_3$, and an intense blue colour with 2:6-dichloroquinone chloroimide in the presence of Na_2CO_3.

The glucuronide could be one of two compounds, 4-(β-D-glucuronosido)-2-hydroxyacetophenone (V)
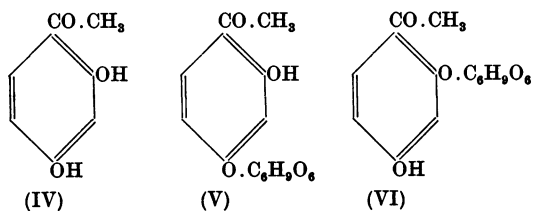
or 2-(β-D-glucuronosido)-4-hydroxyacetophenone (VI), depending on which hydroxyl group had been conjugated.

The procedure used to establish which of these two possibilities was the correct one was similar to that described in earlier papers in this series (Dodgson & Williams, 1949), and consisted of partial methylation of the glucuronide to give either (VII) or (VIII). Subsequent hydrolysis of VII would give 4-hydroxy-2-methoxyacetophenone (IX), whereas hydrolysis of VIII would give 2-hydroxy-4-methoxyacetophenone (X). Both these compounds are readily characterizable.

(VII)   (VIII)
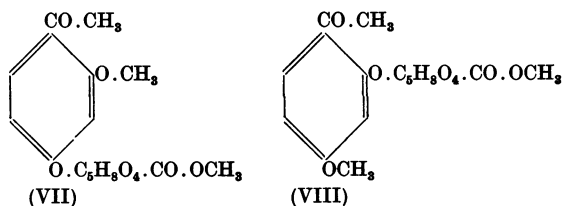
The pure glucuronide was methylated (as a suspension in ethanol) with ethereal diazomethane until the $FeCl_3$ and 2:6-dichloroquinone chloroimide tests could no longer be obtained. The resultant glucuronide syrup was subsequently hydrolysed with 2N-HCl, and the liberated phenol isolated in the usual manner. The crystalline product obtained was identified as 4-hydroxy-2-methoxyacetophenone (IX), by comparison with a pure sample of the compound prepared synthetically; m.p. and mixed m.p. 137–138°; m.p. and mixed m.p. of the corresponding *p*-toluenesulphonate derivative, 121·5°. 2-Hydroxy-4-methoxyacetophenone (X) melts at 50°, and the corresponding *p*-toluenesulphonate derivative appears to be an oil.

(IV)   (V)   (VI)   (IX)   (X)
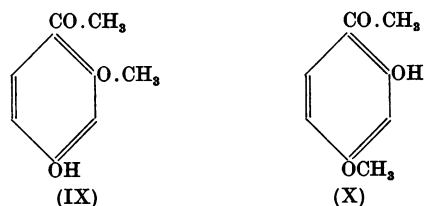
The structure of resacetophenone glucuronide is therefore established as 4-(β-D-glucuronosido)-2-hydroxyacetophenone (V). Resacetophenone is thus behaving similarly to the dihydroxychloro-benzenes as regards the orientation of glucuronic acid conjugation. The hydroxyl group which is farthest away from the substituent group (Cl or —COCH₃) is preferentially conjugated.

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Organic Catalysts of the Oxidation of Ascorbic Acid and of Ketoses. By J. PRYDE. (*Physiology Institute, Newport Road, Cardiff*)

Two of the possible metabolites of 2:4:6-trinitrotoluene (T.N.T.), namely, 2:4:6-trinitrobenzoic acid and 2:4:6-trinitrobenzyl alcohol (Channon, Mills & Williams, 1944), catalyse the oxidation of ascorbic acid by molecular oxygen at pH 7·4, both in the presence and in the absence of surviving mammalian tissue. The oxidation has been followed by the Warburg manometric technique and by titration and colorimetric techniques (Musulin & King, 1936; Roe & Oesterling, 1944), both in the presence and in the absence of inhibitors of metallic ion catalysts such as sodium diethyl dithiocarbamate, 8-hydroxyquinoline and cyanide. The reaction catalysed is:

$$C_6H_8O_6 + O_2 = C_6H_6O_6 + H_2O_2,$$
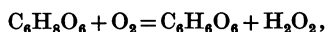
since, in the presence of ox-liver catalase (impregnated filter-paper strips), the measured oxygen uptake is halved. The catalysis is a true organic one and therefore resembles that described by Snow & Zilva (1942), who observed the catalysis of ascorbic acid oxidation by tea infusions and by gallic acid, and whose observations on gallic acid have been confirmed in the course of the present investigations.

No catalytic effect has been observed with T.N.T., itself, nor with certain compounds derived from or closely related to T.N.T., for instance, 2:6-dinitro-4-hydroxylaminotoluene, 2:6-dinitro-p-toluidine, 2:4:6-trinitro-m-cresol and 4:6-dinitro-o-cresol. Various mono- and dinitrobenzoic acids, mononitrobenzyl alcohols, picric and picramic acids are like-wise negative; so, too, are nicotinic acid, riboflavin and thiamin.

On the other hand, marked catalysis of ascorbic acid oxidation has been observed in the presence of sodium 1:2-naphthoquinone-4-sulphonate and of 1:4-naphthoquinone which themselves show only a negligible oxygen uptake at pH 7·4. As already stated T.N.T. itself has no catalytic effect but the isomeric 2:4:5-trinitrotoluene acts as a catalyst, an observation possibly related to its ease of hydrolysis to 2:4-dinitro-5-hydroxytoluene (Brownlie & Cumming, 1946). Another polynitro substance which acts as a catalyst is 1:2:3-trinitronaphthalene. It seems possible that a considerable number of organic compounds may act as catalysts of the aerobic oxidation of ascorbic acid.

Meanwhile, it has now been observed that the oxidation *in vitro* of the ketoses fructose and sorbose in alkaline phosphate media (pH 11·5) by molecular oxygen is also strongly catalysed by 2:4:6-trinitrobenzoic acid and by 2:4:6-trinitrobenzyl alcohol but not by gallic acid. Sodium 1:2-naphthoquinone-4-sulphonate, which itself shows but slight oxygen uptake at pH 11·5 in the medium used, gives marked catalysis of fructose oxidation. In the same alkaline phosphate media 2:4:6-trinitrobenzyl alcohol shows some catalytic effect on the rates of oxidation, much lower than those of the ketoses, shown by the aldoses glucose, mannose and galactose.

Work continues with the hope of elucidating the mechanism and possible significance of these catalytic effects.

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Kinetic Studies of the Metabolism of Foreign Organic Compounds. By H. G. BRAY, W. V. THORPE and K. WHITE. (*Physiology Department, Medical School, University of Birmingham*)

Certain radicals in aromatic compounds undergo characteristic reactions in the body. Most of these reactions fall within the following classification:

(1) Conjugation.

(2) Conversion of potential to actual centres for conjugation.

(3) Introduction of new centres for conjugation.

Compounds containing such radicals have more than one possible fate, and investigations in this laboratory into the metabolism of these compounds have shown that in general, while more than one reaction occurs, conjugation is preferred to formation of conjugable groups, and conversion of potential centres to introduction of new ones (see Thorpe, 1950).

The relative amounts of metabolites and the time taken for excretion will depend on the velocities of these reactions. Consequently, a series of investigations has been planned in which it is hoped to determine the velocities of typical metabolic reactions which foreign organic compounds may undergo in the rabbit. In this first investigation the formation of benzoic acid from toluene, benzyl alcohol, benzaldehyde and benzamide and its conjugation with glycine and glucuronic acid have been studied.

It has been shown that all but the hippuric acid synthesis are first order reactions, for which the velocity constants have been determined. The rate of conjugation with glycine is constant. It had previously been observed that the ratio of glycine conjugate to glucuronide excreted varied with the compound fed and the dose level (Bray, Neale & Thorpe, 1946; Bray, Humphris & Thorpe, 1949). The present findings show that this ratio is a function of the velocity constants and the dose level. In addition the values of the velocity constants help to elucidate the mechanism of the oxidation of toluene.

The experimental method essentially comprised the analysis of blood samples, and of urine samples timed and collected by means of a fraction-collector.

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Further Observations on the Use of Paper Chromatography for the Detection of Phenolic Compounds in Metabolic Studies. By H. G. BRAY, H. J. LAKE, W. V. THORPE and K. WHITE. (*Physiology Department, Medical School, University of Birmingham*)

Methods for the detection by paper chromatography of the phenolic compounds encountered in metabolic studies of the hydroxybenzoic acids and amides (Bray, Thorpe & White, 1950a) and of cresols (Bray, Thorpe & White, 1950b) have already been described. In subsequent work the following solvent mixtures have proved of value: (1) light petroleum (b.p. 40–60°) saturated with formic acid (98–100 %) for fast-moving compounds, (2) benzene saturated with formic acid which gives better separation than the benzene-acetic acid-water mixtures used previously, (3) chloroform-acetic acid-water mixtures for slow moving compounds. A mixture of n-butanol (4 vol.), pyridine (8 vol.), saturated aqueous NaCl (5 vol.), ammonia, sp.gr. 0·880 (3 vol.), gives better separation (e.g. of o-, m- and p-hydroxybenzoic acids) than the original mixture of Evans, Parr & Evans (1949). Preliminary experiments with columns of paper powder (cf. Hough, Jones & Wadman, 1949) have given encouraging results in the isolation of urinary phenols.

It has now been shown that the phenolic acid of normal rabbit urine previously assumed to be mainly p-hydroxybenzoic acid (Bray et al. 1950a) is a mixture of both m- and p-acids, the former predominating (cf. Lederer & Polonsky, 1948). The amount of the m-isomer is not significantly increased by administration of benzoic acid.

Both 4-amino-3-hydroxybenzenesulphonic acid and its amide have been identified as hydrolysis products of the glucuronide fraction of urine from rabbits dosed with sulphamezathine.

In earlier studies of the metabolism of amino- and nitro-benzamides (Bray, Lake, Neale, Thorpe & Wood, 1948; Bray, Thorpe & Wood, 1949) we were unable to identify by isolation all the hydroxylation products which would be expected theoretically. Paper chromatograms provide clear evidence of the presence of 3-amino-2-hydroxy-, 3-amino-4-hydroxy- and 3-amino-6-hydroxybenzoic acids in the hydrolysed urine of rabbits dosed with m-aminobenzamide, and of 2-amino-3-hydroxy- and 2-amino-5-hydroxybenzoic acids from rabbits dosed with o-nitrobenzamide. 2-Amino-5-hydroxybenzoic acid has also been detected as a metabolite of anthranilic acid.

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Spectrophotometric Determination of β-Glucuronidase Activity using *p*-Chlorophenylglucuronide as Substrate. By B. Spencer and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

p-Chlorophenylglucuronide (monohydrate; m.p. 151°, $[\alpha]_D - 82\cdot5°$ in water) is a compound which can be made biosynthetically in good yield (1·1 g./g. *p*-chlorophenol) by direct ether extraction of the 12 hr. urine of rabbits receiving *p*-chlorophenol orally (0·5 g./kg.). Furthermore, small amounts of *p*-chlorophenol can be easily determined as its anion with the Unicam spectrophotometer in the presence of relatively large amounts of *p*-chlorophenylglucuronide. The ultraviolet absorption spectra of *p*-chlorophenol and its glucuronide in acid and alkali are given in Table 1. It is clear from this table that, in alkali, *p*-chlorophenol has a totally different spectrum from its glucuronide and hence by measuring the extinction at 245 mμ. (or in strong solutions at 298 mμ.) *p*-chlorophenol can be estimated in the presence of its glucuronide. *p*-Chlorophenylglucuronide has only a small absorption at these wavelengths, i.e. at 245 mμ., ϵ is 70 and at 298 mμ., ϵ is *c.* 20. In the dilutions used in our experiments, 5 μg. of *p*-chlorophenol were readily estimated. The extinctions at 245 and 298 mμ. obey Beer's law and are not affected by the acetate buffer and ethanol used in the assay.

The method was applied to an active β-glucuronidase extract prepared from ox-spleen according to Talalay, Fishman & Huggins (1946) and fractionated according to Mills (1948). For the estimation protein, which has a significant absorption at these wavelengths, was removed with ethanol (80 % final concentration).

Using this method pH optima were demonstrated at pH 4·1 and 5·2 in acetate buffer, and the activity of the enzyme was shown to be proportional to time and concentration of the enzyme. These and other results were discussed.

Table 1

	In 0·1 N-HCl		In 0·1 N-NaOH	
	mμ.	$\epsilon_{max.}$	mμ.	$\epsilon_{max.}$
p-Chlorophenol	222	8,900	245	11,650
	280	1,600	298	2,600
p-Chlorophenyl-glucuronide	220	10,700	219·5	10,800
	275·5	910	276	970

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An Ether-Soluble Precursor of Coproporphyrin in Urine. By D. N. Raine (introduced by F. K. Herbert). (*Department of Chemical Pathology, The Medical School, King's College, Newcastle upon Tyne*)

When freshly passed human urine is treated with acetic acid and extracted with ether, it is found that the ether extract contains both preformed coproporphyrin, which can be immediately extracted by 1 % HCl (w/v), and a residual non-fluorescent substance which yields fluorescent porphyrin when the ether extract is allowed to stand in daylight. The solubilities of this porphyrin are those of coproporphyrin. When the urine is passed in the dark, and handled in a dark room up to the stage of extraction of the preformed coproporphyrin with HCl, and the ether layer then exposed to daylight, the yield of preformed coproporphyrin is less, and the yield of total coproporphyrin greater, than when the pro-

cedure is carried out entirely in daylight. Any exposure of urine to light before extraction leads to losses. Normally the major fraction of urinary coproporphyrin is in the form of precursor.

The transformation of precursor to porphyrin has been studied with a view to defining the chemical reaction and developing a satisfactory quantitative method for the estimation of total coproporphyrin. Evidence has been obtained that the precursor is a reduced form of the porphyrin, possibly identical with the porphyrinogen obtained by Fischer & Orth (1937) by reduction of porphyrin.

The method of exposing the ether extract to light after removal of preformed coproporphyrin can be used for quantitative estimation of the precursor, but the time required for the conversion is 24 hr. in summer light, and 3–4 days in winter. Various oxidants have been employed to accelerate the conversion and the best is quinhydrone added to the

ether extract in a final concentration of approximately 0·04 %. In daylight with quinhydrone the maximum yield is obtained in 2 hr.

The standard values for normal coproporphyrin excretion in man require revision in the light of these findings. Preliminary estimations indicate a normal total urinary excretion of the order of 200 μg./day.

When freshly passed urine is treated by the adsorption technique of Sveinsson, Rimington & Barnes (1949), preformed coproporphyrin is adsorbed on the calcium phosphate precipitate and the precursor is not adsorbed.

The administration of ascorbic acid to normal subjects, to the point of saturation, leads to the excretion of an increased proportion of the precursor. This is in accord with the conception that the precursor is the reduced form of the porphyrin.

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The Distribution of Fructose in Fractionated Specimens of Human Semen. By J. PRYDE.

(*Physiology Institute, Newport Road, Cardiff*)

Opportunity has recently been afforded for the examination of a few total ejaculates of human semen which had each been collected in three fractions. Refractive index measurements, sperm counts and fructose determinations are quoted. Two specimens from the same donor gave the figures quoted under A 1 and A 2.

Fraction		Volume (ml.)	$n_D^{20°}$	Sperm (count/ ml. $\times 10^6$)	Fructose (mg. %)
A 1	1	1·2	1·3456	650	120
	2	1·4	1·3496	205	530
	3	1·2	1·3540	75	906
A 2	1	1·0	1·3468	810	84
	2	1·3	1·3510	120	560
	3	1·2	1·3550	40	1016
B	1	0·8	1·3460	612	212
	2	1·2	1·3468	255	368
	3	2·5	1·3467	120	604

These figures show unusually sharp demarcation of the prostatic-spermatic and the vesicular com-

ponents of the fluid (MacLeod & Hotchkiss, 1941; Gutman & Gutman, 1941) and strongly support the view that the fructose of human semen is provided by the seminal vesicles (Huggins & Johnson, 1933; Mann, 1946). Mann (1946) has commented on the variability of the fructose concentrations in semen of the same species. The fructose values for both fractions 3 of A 1 and A 2 suggest that, with strikingly high vesicular figures of the order here found, variations in the amount of the vesicular component included in the ejaculate could effect considerable fluctuations in the final fructose concentration of the whole semen.

The pattern above described, with its well-marked separation of components, cannot be regarded as of general occurrence, at least to the same degree. As an example, figures are quoted for specimen B from a different donor. In particular, the closeness of the three refractive index readings in B contrasts strongly with the considerable differences shown by the corresponding readings for A 1 and A 2.

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Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. Davidson,
I. Leslie and J. C. White. (*From the Department of Biochemistry, University of Glasgow, and the
Department of Pathology, Postgraduate Medical School of London*)

In extension of previously reported analyses of the
deoxyribonucleic acid phosphorus (DNAP) and
ribonucleic acid phosphorus (RNAP) content of
aspirated human bone marrow (Davidson, Leslie &
White, 1947, 1948), we now report a modification
involving enumeration of the nucleated cell content
of the samples analysed. Results are expressed in
terms of DNAP and RNAP per cell (Table 1), and
are average values for the growing and adult cell
populations of the analysed samples. The recent
results of Vendrely & Vendrely (1948, 1949) and of
Mirsky & Ris (1949) suggest a striking constancy in
the DNAP content of normal cell nuclei from the
tissues of any given species, and our figures for
DNAP are of the same order as those quoted by the
Vendrelys for human liver nuclei.

There is no significant difference between the
means for the normal and the leukaemic series, either
as a whole, or considering only acute leukaemia prior
to therapy.

A small series of 6 cases of iron-deficiency anaemia
has not shown significant variation of the mean
DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and
other megaloblastic anaemias are shown in Tables 2
and 3.

It must be noted clearly that the group under

therapy cannot be considered as returned to normal,
either as regards blood picture, marrow cytology or
adequacy of therapy. The significant fall in RNAP
from that in the group prior to therapy parallels the
general increase in maturity of the marrow under
therapy. Cases fully treated and returned to normal
are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in
μg. $\times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/ DNAP
Mean	8·54	6·33	0·75
s.e. of obs.	2·89	3·03	0·326
Observed range	4·0–15·0	2·1–13·5	0·43–1·9

*Marrow from cases of leukaemia of various types,
before and during therapy*

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8·75	7·59	0·90
s.e. of obs.	3·05	3·72	0·30
Observed range	3·9–17·4	2·6–17·4	0·3–1·8

Table 2. *Cases of pernicious anaemia and other megaloblastic anaemias*

NAP in μg. $\times 10^{-7}$ per cell

		DNAP 28 obs. on 12 cases	RNAP	Ratio DNAP/RNAP 28 obs. on 13 cases
Group as a whole	Mean	12·6	10·9	0·87
	s.e.	4·56	5·03	0·27
	Observed range	6·6–22·8	2·3–25·1	0·35–1·5
		12 obs. on 12 cases	11 obs. on 11 cases	12 obs. on 12 cases
Group prior to therapy	Mean	12·57	13·38	1·06
	s.e.	4·17	5·19	0·249
	Observed range	8·1–22·8	7·5–25·1	0·69–1·5
		17 obs. on 8 cases	15 obs. on 8 cases	16 obs. on 9 cases
Group during the course of therapy	Mean	12·63	9·09	0·73
	s.e.	4·36	4·21	0·198
	Observed range	6·6–18·8	2·3–17·6	0·35–1·0

Table 3. *t test of significance between means*

		DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	P	<0·001	<0·001	0·2–0·1
	Degrees of freedom	44 Highly significant	44 Highly significant	46 Not significant
Megaloblastic series before therapy compared with normal	P	0·01–0·001	<0·001	0·01–0·001
	Degrees of freedom	28 Highly significant	29 Highly significant	30 Highly significant
Megaloblastic series during therapy compared with normal	P	0·01–0·001	0·05–0·02	0·8–0·7
	Degrees of freedom	33 Highly significant	33 Significant	34 Not significant
Megaloblastic series before and during therapy compared	P	0·7–0·6	0·05–0·02	<0·001
	Degrees of freedom	27 Not significant	24 Significant	26 Highly significant

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. Buffa, W. D. Lotspeich, R. A. Peters and R. W. Wakelin. (*Department of Biochemistry, University of Oxford*)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluoro-tricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoro-acetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalo-succinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate dis-appearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

Table 1. *Rat heart aconitase*

	Citric acid (μmol.)	
Time (min.) ...	0	60
Additions:		
cis-Aconitate (5 μmol.)	0·21	3·90
cis-Aconitate + 'active' fraction	0·08	3·96
Citrate (5 μmol.)	4·90	4·34
Citrate + 'active' fraction	5·27	4·38

Table 2. *Pig heart isocitric dehydrogenase*

	$E_{340\,m\mu.}$ (max. value)
DL-*iso*citrate only	0·076–0·065
Same + 'active' fraction	0·075
Same + p-chloromercuricbenzoic acid 1.33×10^{-5} M	0·004

Table 3. *Pig heart oxalosuccinic decarboxylase*

(CO_2 evolution from 10 μmol. oxalosuccinate in 30 min. at 13·5° C. Net values)

	CO_2 (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-*iso*citrate (control)	14

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Metabolic Maintenance of the Inorganic and Creatine Phosphates of Brain Tissue *in vitro*. By H. McILWAIN and J. D. CHESHIRE. (*Biochemical Laboratories, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

The phosphates of mammalian brain change markedly in concentration with changed activity of the central nervous system (for a recent assessing see McIlwain, 1950). Inorganic phosphate and creatine phosphate are among the constituents most sensitive to the actions of convulsants (Stone, Webster & Gurdjian, 1945; Klein & Olsen, 1947) and narcotics (Stone, 1940; Le Page, 1946). To investigate the actions of such substances we desired to study their actions on separated brain tissue. The inorganic and creatine phosphates of brain are, however, found to undergo extremely rapid change after death of animals. Fall in phosphocreatine to 30 % of the original value in 3 sec. has been observed in dogs (Kerr, 1935) and to 25 % in mice on decapitation (Stone, 1940). About a threefold increase in inorganic phosphate occurred concomitantly. This contrasts with observations on the balance of products of carbohydrate metabolism in brain slices and on the rate of such metabolism. Here the findings, some time after death, can be very close to those determined by arterial-venous difference (Schmidt, Kety & Pennes, 1945).

We have now observed in guinea-pig brain, a post-mortem fall in creatine phosphate and rise in inorganic phosphate comparable to those described above. However, we have found that after respiration of brain cortex slices in balanced salines with substrates, the phospho-creatine of the slices greatly increased, and their inorganic phosphate fell. With suitable mixtures, values approaching those normal to the tissue *in vivo* could be reached. In addition to glucose, glutamate markedly affected the values reached. The findings regarding the maintenance of the phosphates of brain tissue *in vitro* thus parallel those of Krebs & Eggleston (1949) regarding its maintenance of potassium salts. Phosphates were determined in our experiments after separation by Ca-ethanol precipitations. The creatine phosphate precipitated was estimated after conversion to creatinine; it is differentiated by the conditions of precipitation, from creatine present as such.

Estimation of brain phosphates has been applied in studying the action of narcotics. By determining the inorganic phosphate, phosphocreatine and lactic acid during *in vitro* metabolism by brain cortex, it has been shown (Buchel & McIlwain, 1950) that the inhibition of respiration associated with the action of narcotics *in vitro* (see for example Quastel & Wheatley, 1933; Quastel, 1943) has very different characteristics from that previously found (Stone, 1938, 1940; Richter & Dawson, 1948) to be associated with their action *in vivo*.

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The Biosynthesis of Choline Methyl Groups in the Rat. By H. R. V. ARNSTEIN. (*The National Institute for Medical Research, Mill Hill, London, N.W.* 7)

After feeding ^{14}C-methyl-labelled choline to rats the isotope was found both in the respiratory CO_2 and in the β-carbon atom of serine (Sakami, 1949). Since the β-carbon atom of serine can arise by the *in vivo* condensation of formate and glycine (Sakami, 1948) it seems likely that the first step of the oxidation of choline methyl groups is the formation of a 1-carbon fragment, probably formate or formaldehyde.

Although the supply of labile methyl groups to the rat, either as choline or methionine, is believed to be essential, it seemed possible that the animal is able to synthesize a part of its requirement from other sources. In this connexion it was of interest to examine the reverse reaction, i.e. the reduction of formate and similar potential 1-carbon fragments to choline methyl groups.

The isotopically-labelled compounds listed in Table 1 were fed to adult rats kept on a stock diet. After a time interval of between 1 and 5 days the animals were killed. The choline was isolated as reineckate, converted to the chloroplatinate and degraded to trimethylamine chloroplatinate (du Vigneaud, Cohn, Chandler, Schenk & Simmonds, 1941). The results of these experiments are given in Table 2. All the compounds except carboxyl-labelled glycine gave rise to isotopically labelled choline, containing isotope in both the ethanolamine and methyl groups. It is concluded that the 1-carbon fragment derived from the β-carbon atom of L-serine and the α-carbon atom of glycine, as well as methanol and formate, can be converted to choline methyl groups by the intact rat. (A similar experiment with ^{14}C-methanol has recently been reported by du Vigneaud & Verly (1950). CO_2, on the other hand, is not reduced, as carboxyl-labelled glycine was not a precursor of choline methyl groups. These results further show that the conversion of glycine to choline, which has previously been demonstrated with ^{15}N by Stetten (1941, 1942), takes place through serine with loss of the carboxyl group.

Table 1

Exp. no.	No. of rats	Body wt. (g.)	No. of days kept before killing	Labelled compound	Wt. fed (mg.)	Radioactivity (μC.) or atom % excess ^{15}N
1	2	508	2½	D-β-^{14}C-serine	142·6	11·7
2	2	550	2½	L-β-^{14}C-serine	122·0	10·0
3	1	302	3	α-^{14}C-glycine	9·6	9·6
4	1	260	1	^{14}C-methanol	13·7	10·3
				^{15}N-glycine	10·0	30·2
5	1	387	3	^{14}C-sodium formate	41·7	22·3
				^{15}N-glycine	10·0	30·2
6	2	590	4	Carboxyl-^{14}C-glycine	45·6	20·5
				^{15}N-glycine	53·0	30·2

Table 2

Exp. no.	Labelled compound fed	Radioactivity (10^{-3} μC./mM.)		Dilution factor*		% radioactivity in trimethylamine
		Choline	Trimethyl-amine	Choline	Trimethyl-amine	
1	D-β-^{14}C-serine	Inactive	—	—	—	—
2	L-β-^{14}C-serine	9·89	2·08	184	878	21·0
3	α-^{14}C-glycine	9·31	1·70	341	1866	18·2
4	^{14}C-methanol	13·86	5·52	286	719	39·8
	^{15}N-glycine	—	—	172	—	—
5	^{14}C-sodium formate	22·66	4·52	254	1278	19·9
	^{15}N-glycine	—	—	174	—	—
6	Carboxyl-^{14}C-glycine	Inactive	—	—	—	—
	^{15}N-glycine	—	—	302	—	—

* The dilution factor given is the reciprocal of the 'concentration coefficient' as defined by Anker (1948). It gives the dilution of isotope which would have been found if exactly 1 mM./100 g. body wt. had been fed.

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The Effects of Alcohols on Cholinesterases. 1. General. By K. P. FELLOWES, J. P. RUTLAND and A. TODRICK

Alcohols affect the hydrolysis of 0·018M-acetylcholine by rat brain in two different ways. At low concentrations they cause activation but above an optimum concentration the activation rapidly falls off and with increasing alcohol concentration complete inactivation occurs.

The optimum concentration is approximately

The alcohol concentrations involved are probably sufficiently high for the latter effect to be due to denaturation.

The effect of n-butanol on the activity of different enzyme preparations against the three standard substrates has been examined. With one exception all show activation:

Enzyme	Substrate	Maximum activation (%)
Rat brain	Acetylcholine (0·018 M)	110
Human erythrocyte	Acetylcholine (0·018 M)	55
Horse serum	Benzoylcholine (0·006 M)	28
Rat brain	Acetyl β-methylcholine (0·028 M)	14

halved for each additional carbon atom in the alcohol. The degree of activation is maximal with n-butanol and falls off rapidly on either side.

n-Butanol has been studied in some detail. The activation appears to be almost instantaneous and is completely reversible by dialysis. The inactivation is progressive and appears to have a high temperature coefficient. It is not reversible by dialysis.

The optimum activating concentrations are close to 0·2M (somewhat higher with the brain-acetylcholine system).

The hydrolysis of acetylcholine by serum cholinesterase is inhibited by butanol. The inhibition occurs in the concentration range in which the other systems are activated and increases progressively with butanol concentration.

The Effect of Alcohols on Cholinesterases. 2. Investigation of the Mechanism of Activation. By K. P. FELLOWES, J. P. RUTLAND and A. TODRICK

The results quoted in the previous paper and, in particular, the exceptional behaviour of the serum cholinesterase-acetylcholine system have suggested a cause for the activation. According to Augustinsson (1948) this latter system is the only one of the five examined in which inhibition of the enzyme by excess substrate does not occur. The theory is advanced that alcohols interfere with the inhibition by excess substrate, the net result being an activation.

This has been tested by varying the substrate concentration. If the hypothesis is correct, the activation should disappear at substrate concentrations below the optimum.

The inhibition of the serum cholinesterase-acetylcholine system has been found to obey the equation of Lineweaver & Burk (1934) for reversible competitive inhibition though there is some deviation at the lowest concentrations.

The optimum substrate concentrations for the serum cholinesterase-benzoylcholine and brain cholinesterase-acetylcholine systems are low and there are considerable practical difficulties in working below them. However, in the latter case, it has

been possible to demonstrate a shift in the optimum from pS 2·6 to 2·35 due to 0·19M-n-butanol and a falling off in activation from 65% at pS 2·0 to a negligible value at pS 2·9.

The system brain cholinesterase-acetyl β-methylcholine is much more satisfactory to work with since it has a pS optimum of 1·8–2·0. It has been possible to show that n-butanol causes 20–30% activation at pS 1·0–1·5 but that at pS 2·4 and 2·7 there is an inhibition of the same value.

This result is in accordance with the hypothesis advanced. Augustinsson (1949) has already suggested that the inhibition caused by a competitive reversible inhibitor will depend on the substrate concentration. However, it does not appear to have been suggested before that the use of super-optimal substrate concentrations could result in inhibitors bringing about apparent activations.

The use of such high substrate concentrations has been not uncommon. It is not suggested that this will have been a major source of error except where competitive reversible inhibitors are involved.

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The Turn-over Number of Cholinesterases. By W. K. Berry (introduced by D. R. Davies)

The turn-over number (T.N.) of cholinesterase (ChE) cannot be expressed in terms of molecules of enzyme because samples pure enough for molecular weight determinations are generally not available. A T.N. in terms of active centres can be obtained comparatively easily if certain assumptions are made, for all of which there is some experimental support. These are that:

(1) there is a bimolecular reaction between active centres and an inhibitor,

(2) inhibition is irreversible,

(3) the inhibitor reacts practically exclusively with ChE.

When the inhibition reaction is allowed to go to completion, the loss in enzyme activity is directly proportional to the quantity of inhibitor added.

Dicyclohexyl fluorophosphate (DCFP) and diethyl p-nitrophenyl phosphate (E 600), both potent inhibitors of ChE, have been used because they are extremely resistant to hydrolysis. A fresh sample of E 600 was used as a check on the DCFP, which was over a year old. Both gave the same T.N. with partially purified human erythrocyte ChE, viz. 162,000 molecules ACh per minute. This was taken as evidence that the DCFP had not deteriorated. It is the better inhibitor because it is more stable and reacts more rapidly.

A comparison of crude and 30-fold purified human erythrocyte ChE showed that the probability that the two estimations were identical was 0·3. This finding, that a good value of T.N. could be obtained from unpurified material, enabled a species survey to be carried out without the necessity of purifying each sample. It would nevertheless apply only to such tissues as can be shown to contain predominantly or exclusively one ChE.

The following provisional results are given for the blood of different species:

Species	Plasma	Cells
Human	48,000	171,000
Horse	94,000*	13,000
Goat	—	53,000*
Dog	99,000	50,000
Rat	—	10,000

* Partially purified.

In addition to differences in T.N., there were some differences in the rate of inhibition, that of dog cells being slowest.

It is not yet possible to say whether the species differences observed are due to the existence of different enzymes, or to differences in the environment of the same basic types.

The Variation of the Cholinesterases of the Blood in Health. By D. R. Davies and J. P. Rutland

Lack of adequate knowledge of the variation of blood constituents in health is a major deficiency in clinical biochemistry. The main reason for this lies in the difficulty, not only in obtaining a sufficient number of blood specimens from healthy people but also in obtaining adequate social and medical data, for the problem is as much statistical and demographic as it is analytical. In this communication the variation in both the red cell and plasma cholinesterases of approximately 200 healthy adults between the ages of 18 and 74 is reported. Three groups were investigated:

(i) A Service group drawn from the R.N., Army and R.A.F., consisting of 72 males between 18 and 30 concerning whom documentary evidence of their standard of physical fitness was forthcoming. They were all of the highest standard of fitness.

(ii) A group of 72 males between 18 and 64 drawn from the general population of south-west England. These were all blood donors and none had any recent history of serious illness.

(iii) A group of 53 females from the same source, aged 25–74.

The mean red cell ChE of the Service group was significantly higher than that of either the male or female groups, between which there was no difference. This difference was not due to age, for there was no difference in either the R.B.C. or plasma enzyme with age. A more detailed analysis suggested that possibly the R.B.C. enzyme did in some way reflect the state of fitness of the individual.

The R.B.C. enzyme varies 30 % about the mean value (this is based upon the usual 95 % fiducial limits), and the plasma enzyme 40 %.

Technique	Cells		Plasma	
	No. in group	Coeff. of variation	No. in group	Coeff. of variation
Warburg; acetylcholine as substrate	188	15·7	180	21·2
Warburg; acetyl-β-methylcholine as substrate	50	15·4	—	—
Warburg; benzoylcholine as substrate	—	—	49	25·8
Electrometric	48	15·9	47	24·8

The coefficient of variation, i.e. the percentage standard deviation, is a valuable parameter for measuring the spread of normal (healthy) levels around the mean, for it is an index whose value is independent of the technique of measuring the enzyme. Three methods were used for determining ChE activity of various specimens, and the coefficients of variation were determined. They were essentially the same, irrespective of the method.

The slightly wider values in the case of the plasma may be due to the fact that the plasma is less homogeneous with reference to its ChE content.

The Electrometric Method of Michel for the Estimation of Cholinesterases. By D. R. Davies and J. P. Rutland

It is often necessary in routine investigations to perform cholinesterase estimations in large numbers in a limited period of time. Hitherto, this has been done either by continuous titration or manometrically by Ammon's modification of the Warburg technique. Both these methods are time-consuming and unsuitable for the above type of work.

Recently Michel (1949) described an electrometric method for the estimation of ChE. This consists essentially of a measurement of the rate of fall of pH in weakly buffered solutions, consequent upon the accumulation of acid due to the enzymic hydrolysis of an appropriate choline ester. Since the only apparatus required was a pH meter, a constant temperature bath and suitable test-tubes, and the only observations needed were a limited number of pH determinations, the method seemed particularly suitable for the performance of large numbers of estimations.

The original paper is deficient in experimental proof of a number of issues upon which the method depends, so we have carried out experiments to examine these points. The principle of the electrometric method is that the fall in pH should be directly proportional to the time over a suitably wide range of pH. Michel implies that this condition is sensibly satisfied under the conditions of the estimation, but adduces no data to substantiate it. Furthermore, he himself worked with human red cells and serum only, although later workers in the same laboratories have quoted results using goat and dog bloods, and also for brain. We have followed the fall in pH using R.B.C. and plasma of various species and homogenates of rat tissues (see Fig. 1).

We have also determined the relationship between enzyme concentration and activity in goat cells diluted with water and buffer and also plasma diluted with inactivated plasma. Provided the activity was not less than 0·1 Δ pH/hr. (Michel defines the unit of enzyme activity as the rate of fall of pH/hr., i.e. Δ pH/hr.), then a strict proportionality between enzyme concentration and activity was observed.
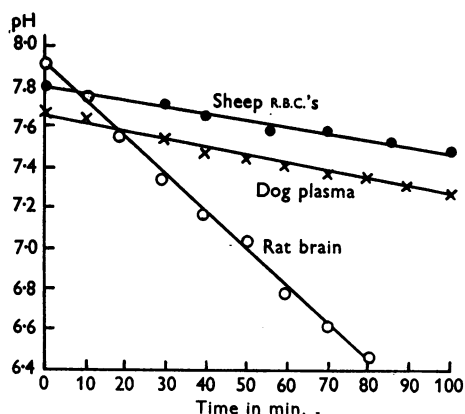

Fig. 1. The variation of pH with time due to cholinesterase in a barbiturate buffered system with acetylcholine as substrate. Similar curves have been obtained for:

Plasma	R.B.C.	Tissues (rat)
Rabbit	Rabbit	Liver
Dogs	Dogs	Heart
Goat	Goat	
Human	Human	
Rat	Sheep	

Dilution = 1–50.

Correlation between the electrometric and Warburg methods, which was tested not only upon diluted enzyme preparations but also upon various specimens of human cells and plasma, was good.

This method is quite satisfactory for many problems involving the routine estimation of ChE in

R.B.C.'s, plasma and tissues, provided that such tissues contain reasonable amounts of enzyme, i.e. it is not suitable for estimating the very small amounts which may remain after the administration of anti-ChE drugs. In fairness, however, it should be pointed out that it is no worse than the manometric method in this respect.

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DEMONSTRATIONS

A Bridge Unit for Filter-Paper Chromatography. By E. Kawerau. (*Department of Chemical Pathology, St Mary's Hospital, Paddington, London, W. 2*)

The novel feature of this all-glass unit for filter-paper chromatography is the arrangement of the strip in the form of a bridge from one flask to another by passing it through a glass sleeve. The latter is made in two sections for ease of assembly and cleaning. All parts join by standard ground glass joints, the whole unit being made to the author's specifications by Messrs Quickfit and Quartz, Stone, Staffordshire. The overall length of the apparatus is 50 cm.; it can be accommodated in the incubator or the refrigerator as the analytical procedure may demand.

The ground-glass joint in the centre of the sleeve carries two holes to locate with each other, and by rotation it becomes possible either to admit or to exclude air from the apparatus. It has thus been made possible to study the effects of controlled evaporation on band and solvent movement along the filter-paper strip.

The *closed* unit. Reliable R_F values have been obtained for many sugars and they are identical with those obtained by either ascending or descending chromatography employing standard methods; it appears, therefore, that the change in the position of the filter paper in no significant way affects R_F values. Preliminary work on amino-acids and urinary pigments (porphyrins) tends to support this statement.

The *open* unit. This is a hitherto unexplored field of chromatography both from the theoretical and practical aspect. The technique employed at present is to let the filter-paper strip form a complete bridge between two solutions of different composition, one of them containing the substance under investigation. Somewhere in the centre of the strip the two solutions meet and a certain amount of mixing takes place, depending on the composition of the respective solvents. Evaporation provides the driving force for continuation of solvent movement after the paper has become saturated. Where a mixture of pigments is being analysed, they tend to appear in separate zones in which they become gradually more and more concentrated as the supply is being maintained. This method has been found useful in the analysis of urines containing small amounts of pathological pigments, and it should also have a place in preparatory work since it produces concentration as well as separation of the solutes if suitable solvents are chosen.

The advantages of the Bridge unit over existing apparatus are: all-glass construction, small size, interchangeable parts, ease of temperature control, ease of cleaning and the small gas volume which is released when the unit is opened for the removal of the filter-paper strip.

An Automatic Fraction-cutter for Use in Partition Chromatography. By K. S. Dodgson, J. Pryde and A. L. Sims. (*Physiology Institute, Newport Road, Cardiff*)

A unit which is capable of collecting automatically fifty samples at intervals ranging from 1 to 30 min. has been constructed at very low cost. The mechanism is controlled by a variable impulse timer, which has been built from a surplus R.A.F. aerial photography unit. There is no direct mechanical connexion between the motor driving the turntable and the turntable itself, so that the latter can be freely rotated for purposes of adjustment.

The mechanism (Sims, 1938) responsible for moving the turntable has been so designed that additional and larger turntables can be driven simultaneously by the same motor. The apparatus is provided with an automatic stop which can be made to operate at any predetermined time up to 24 hr.

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