SOME ASPECTS OF THE METABOLISM OF THE DEVELOPING CHICK EMBRYO.

The metabolism of 'biologically labile' methyl groups.

A thesis presented for the Degree

of

DOCTOR OF PHILOSOPHY

by

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APPENDIX II/

INTRODUCTION

Excluding inorganic ions and molecules, the methyl group is surely one of the simplest chemical units encountered in the living cell. This radical is found in biological material in covalent combination with various elements, yielding molecules well known to the organic chemist, which could be classified as follows:-

(a) Methyl group attached to nitrogen, as in choline, creatine and adrenaline.

(b) Methyl group attached to sulphur, as in methionine and dimethylthetin.

(c) Methyl group attached to carbon, as in alanine, valine and thymine.

(d) Methyl group attached to oxygen, as in alkaloids such as narcotine.

(e) Methyl group attached to metalloids such as selenium, tellurium and arsenic, yielding selenides, tellurides and arsenides.

Whilst the methylated nitrogen, sulphur and carbon compounds are widely distributed in the plant and animal kingdoms, the methyl ethers and methylated/

methylated metallic complexes are rareties, occurring in certain highly specialized organisms, or under non-physiological circumstances. For this and other reasons to be elaborated later, the subject of biological methylation was for long mainly confined to the study of methyl groups attached to nitrogen or sulphur and was only quite recently extended to include methyl-carbon compounds. The phenomena of synthesis, transport and degradation of the methyl group have occupied the attention of physiologists and biochemists during the greater part of the twentieth century. Although these problems have not been settled, the groundwork has been laid to open the way to a fuller elucidation of the role of the methyl radical in biochemical processes.

History /

History of the biosynthesis of the methyl group.

The first recorded work on the methyl group is probably that of Hofmeister (1894) who made *in vivo* studies on the methylation of tellurium by dogs, and similar experiments *in vitro* with homogenates of organs. Hofmeister concluded that the acquisition of a methyl group was a normal metabolic process, and postulated that the methyl radicals of choline and creatine were in all probability derived from the same source as the methyl groups used in the synthesis of tellurides. He proposed that the methyl groups were transferred when required from a common methylated precursor to the acceptor molecule by means of a 'ferment'.

In the results of Riesser (1913) one finds some confirmation of this point, for although this worker was not interested in methylation studies, he found it was possible to elevate by 15% the creatine content of muscle by means of choline injections. This increase could have been due to the transference of a methyl radical from choline to the creatine precursor, and although the author did not suggest it, this evidence was later put forward in support of the theory that the methyl group/

group could not be produced in the animal body at a sufficient rate to offset the methyl groups lost by catabolism.

In the light of experimental work by Thompson (1917) and others, the 'methyl transfer theory' was supplanted by the hypothesis that methyl groups in the body might originate from formaldehyde.

This worker, using ducks, showed that the creatine output of the birds could be increased by feeding arginine plus paraformaldehyde or hexamethylenetetramine as a source of formaldehyde. Thompson concluded that creatine arose from arginine through the production of glycocyamine, which was then methylated on the ω-nitrogen atom. Creatine could then be formed from this intermediate product by an intramolecular rearrangement.

Thus /

Thus, before the end of the second decade of this century, an experimental approach to the problems of transport and synthesis of the methyl radical had already been made by Hofmeister and Thompson, and it is interesting to note that attention has recently been focussed again on formaldehyde as an intermediate in methyl group metabolism (Sakami, 1950).

Observations as a result of incorporating certain methylated compounds in the diet of the dog and the rat.

The role of the methyl radical in biological processes received its next considerable advance indirectly as a result of the classical researches on the hormone of the pancreas - insulin. Macleod (1924) conducted lengthy studies on insulin-maintained depancreatized dogs extending over several years, and observed on autopsy that all the animals had enlarged yellow fatty livers. In 1930 Hershey, who was collaborating with Macleod, observed that he could cure or prevent this fatty liver condition in these animals by incorporating crude egg yolk lecithin into their diet. Best and Huntsman (1932) demonstrated that the active component/

component in the lecithin was choline, and they also reported that an oxidation product of choline, namely betaine, was effective in a quantitatively lesser degree in preventing the deposition of fat in the liver of the depancreatized animal. To this anti-fatty liver property of choline and betaine they gave the title 'Lipotropic Effect'.

Best and co-workers in 1935 recorded that the protein, casein, free from choline or betaine, was a lipotropic agent, while gelatin was inactive in this respect. Tucker and Eckstein (1937) found that the amino acid methionine also exerted a lipotropic action. They attributed the lipotropic effect of casein to its methionine content, and the inertness of gelatin to the absence of this amino acid.

It thus appeared that the three compounds choline, betaine and methionine had in common this lipotropic action.

Methyl group requirements of the growing white rat.

Rose (1939) and du Vigneaud et al.(1939a) independently commenced studies on the essential nature of the amino acid methionine for the growth of/

of the young white rat, and in this connection they both investigated whether homocystine could replace methionine in the diet of these animals. Homocystine was used in place of homocysteine, the assumption being that the body could cleave each molecule of homocystine to two molecules of homocysteine.

Both these workers fed growing rats on a synthetic diet in which the only sulphur containing amino acid was homocystine; du Vigneaud et al. supplemented the diet of their rats with crystalline thiamine, riboflavin and nicotinic acid, whereas Rose employed a naturally extracted 'vitamin B' supplement obtained from tikitiki (a rice bran preparation) plus milk concentrates. The rats in du Vigneaud's laboratory lost weight steadily, many developed renal lesions and died, but the rats in the experiments conducted by Rose grew steadily although at a sub-optimal rate. In order to reconcile these conflicting results, du Vigneaud examined the vitamin B supplement used by Rose, and found the extract to contain choline.

This finding prompted du Vigneaud et al. (1939b) to try the addition of choline, and its oxidation/

oxidation product betaine, to the rats' synthetic diet. All the animals receiving homocystine plus choline or betaine now grew at the same rate as those maintained with methionine. This proved that methionine could be replaced in the diet by homocystine if choline or betaine was also supplied.

This circumstantial evidence was taken as proof that the rat could not methylate homocysteine unless provided with choline or betaine; however, later experiments by Rose (1941) which were confirmed and extended by Bennett et al. (1943, 1944) demonstrated that young rats could grow sub-optimally on a diet in which the only sulphur containing amino acid was homocystine, using also a synthetic vitamin supplement devoid of choline or betaine. Du Vigneaud et al. (1939b) suggested that one possible explanation of these results lay in refection, or the absorption of products of bacterial synthesis from the gut; they did point out that an occasional animal in their colony could grow sub-optimally on the synthetic diet without choline or betaine.

Many other biologically occurring methylated compounds such as creatine, caffeine, methylamine, etc. were found to be inactive, in so far as these substances/

substances together with homocystine could not replace methionine in the diet of the growing rat. Thus, du Vigneaud et al. (1939b) suggested the name 'Labile Methyl Group' for a methyl group which is capable of intact transference from one molecule to another, as in choline, betaine and methionine. To the process he gave the name 'transmethylation', reviving the 1894 hypothesis of Hofmeister.

Intermediary Metabolism Studies on Transmethylation

Du Vigneaud et al. (1943) conclusively proved the accuracy of this transmethylation concept by feeding animals choline labelled with deuterium in the methyl groups, and subsequently isolating from the carcass methionine with the terminal methyl group labelled with deuterium. This theory also explained the lipotropic action of methionine observed by Tucker and Eckstein (1937) because three molecules of methionine could donate three methyl groups to one molecule of ethanolamine to produce one molecule of choline. This reaction du Vigneaud et al. (1940) subsequently demonstrated by feeding isotopic methionine labelled in the methyl group, and later isolating from the animal body/

body choline which was found to be labelled in the methyl groups.

Following closely on this work, Schoenheimer and Bloch (1940a,b), Borsook and Dubnoff (1940a, b) and du Vigneaud *et al.* (1940, 1941) independently, using isotopic nitrogen and deuterium, demonstrated the *in vivo* and *in vitro* synthesis of creatine from glycine. They were able to show that the intermediate compound in this biological synthesis was not sarcosine but glycocyamine (cf. Thompson, 1917). The final step which involved the methylation of glycocyamine was shown to occur at the expense of a methyl group from choline, betaine or methionine.

In the rat growth experiments previously mentioned, creatine was shown to be inactive as a 'methyl donor', and since the body loses a roughly constant amount of creatine per day, it was recognized that this loss represented a drain on the choline and methionine resources of the body, or as it was termed, a drain on the 'labile methyl group pool'.

In/

In a further extension of their rat growth experiments, du Vigneaud *et al.* (1946) found that the dimethyl analogues of choline and betaine (dimethylaminoethanol and dimethylaminoacetic acid) were relatively inactive as methyl group donors. They therefore proposed that only one methyl group in choline or betaine is 'labile' and hence available in a transmethylation reaction. Betaine had previously been shown by Griffith (1941b) to be only one-third as effective as choline, when assayed in a lipotropic experiment; presumably the conversion of choline to betaine was irreversible and since only one methyl group in betaine was labile, it required three molecules of betaine to provide three methyl groups needed for the synthesis of one molecule of choline, which was probably the ultimate lipotropic agent.

Possible/

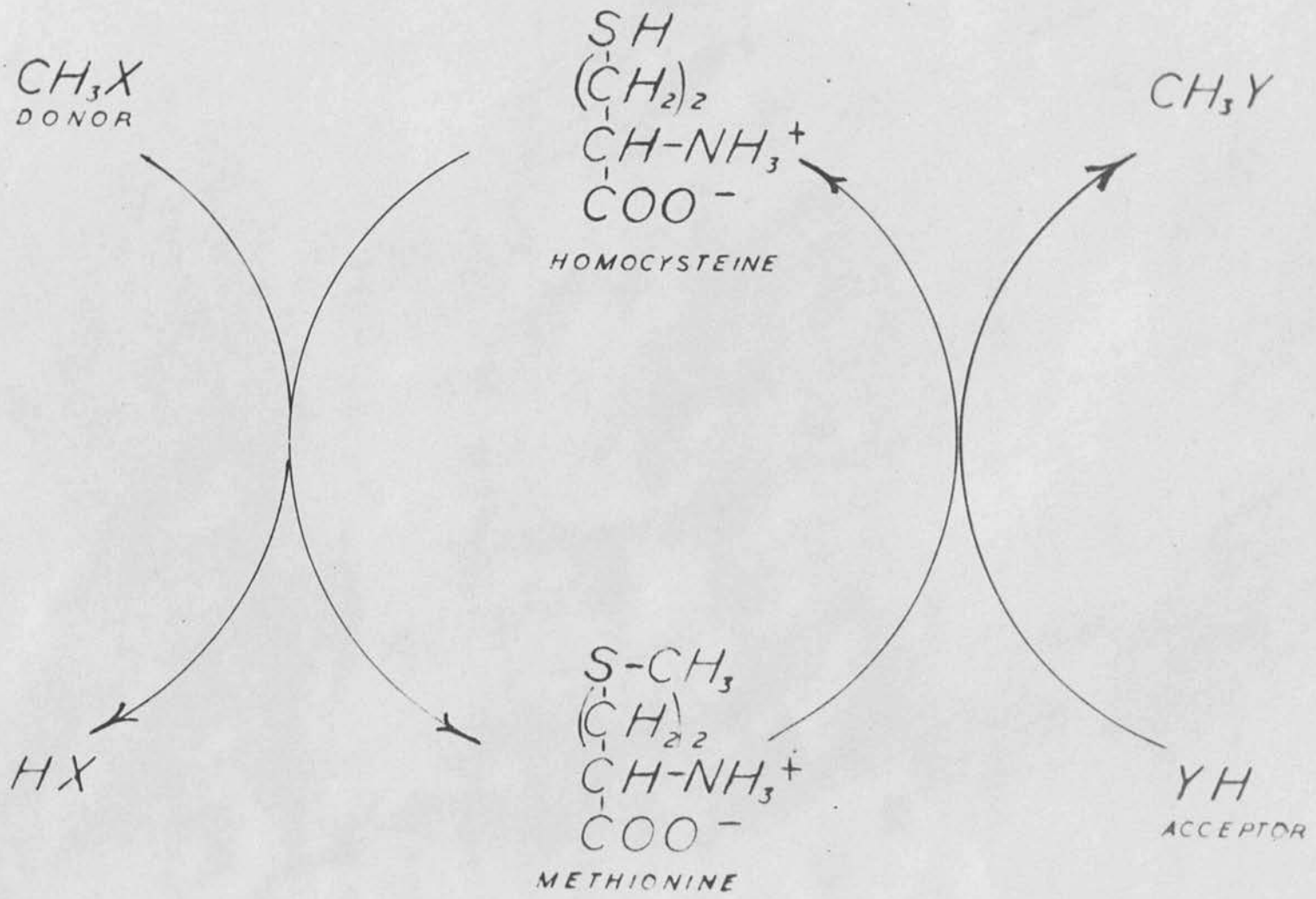

CH3X
DONOR
SH
(CH2)2
CH-NH3+
COO-
HOMOCYSTEINE
CH3Y
HX
S-CH3
(CH2)2
CH-NH3+
COO-
METHIONINE
YH
ACCEPTOR

FIGURE A

Possible Mechanisms involved in the Process of Transmethylation.

Borsook and Dubnoff (1947) studied the rate of transference of a methyl group *in vitro* from choline or betaine to homocysteine in tissue homogenates, and they found that betaine was a more effective donor than choline. They suggested that in all probability choline was oxidized to betaine before acting as a methyl donor. If one accepts the thesis of these workers, then choline and betaine must be on the same metabolic pathway with regard to a transmethylation reaction.

Presumably methionine could also be acting in the same chain of reactions, homocysteine receiving a methyl group from (say) betaine, yielding methionine, which could then donate this methyl radical to another acceptor, thus regenerating homocysteine, as illustrated in Fig. A.

As all transmethylation experiments have been performed by tissue slice or homogenate techniques and never by purified enzyme methods, it is not possible to state whether the transmethylations involve a single donor or several known donors in a definite sequence.

In this connection a further complication arose/

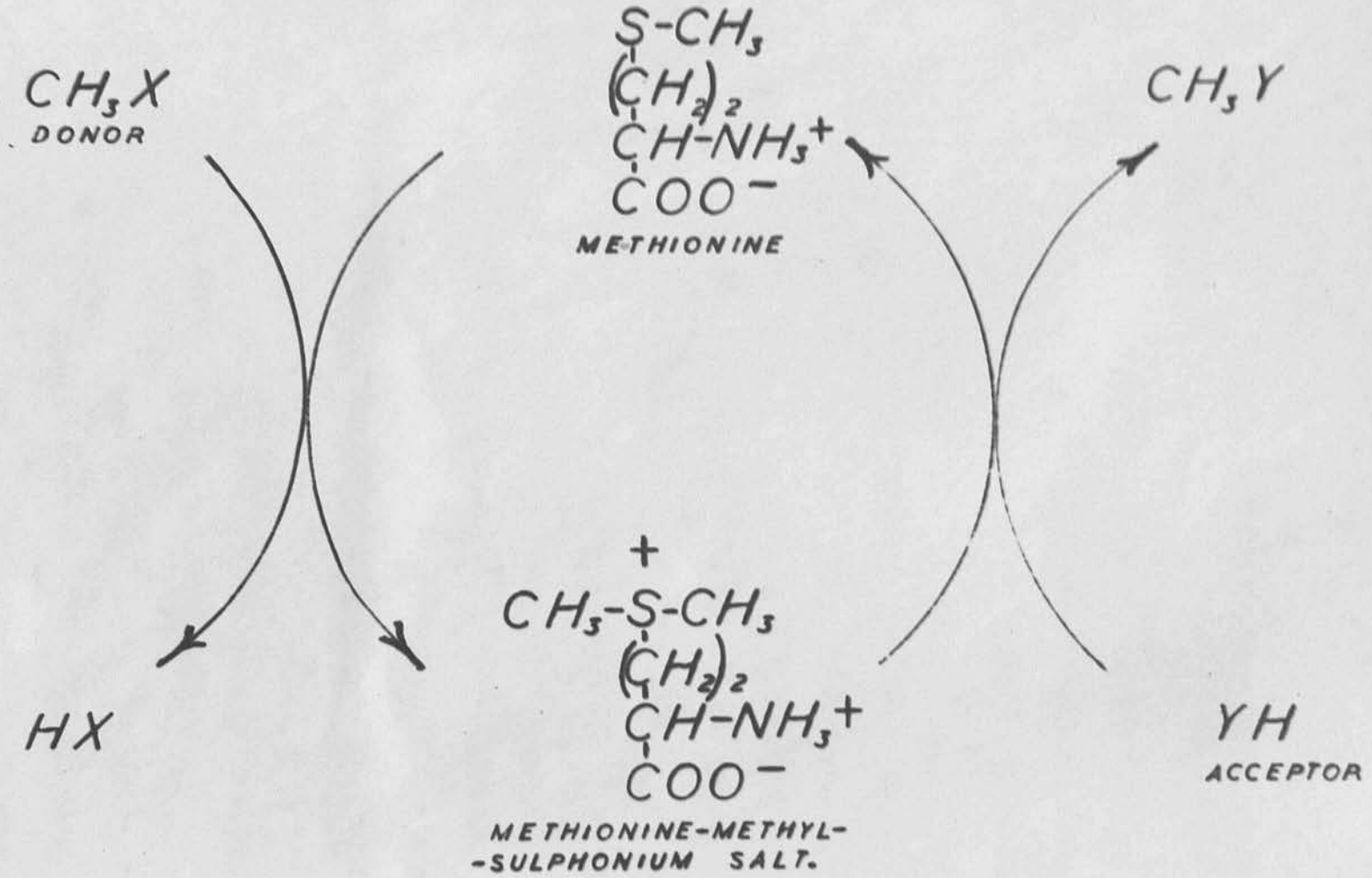

CH3X
DONOR
S-CH3
(CH2)2
CH-NH3+
COO−
METHIONINE
CH3Y
+
CH3-S-CH3
(CH2)2
CH-NH3+
COO−
METHIONINE-METHYL-
-SULPHONIUM SALT.
HX
YH
ACCEPTOR

FIGURE B.

arose when Borsook and Dubnoff (1948) showed that dimethylthetin (the sulphur analogue of betaine) was even more active as a methyl donor than betaine in tissue slice experiments. This substance has not been detected in nature, but Challenger (1947) found its homologue dimethyl-β-propiothetin in a marine alga; therefore the observations of Borsook and Dubnoff on the activity of dimethylthetin as a methyl donor should be given some consideration. For example the possibility has not yet been excluded that the 'thetin' of methionine, (methionine-methyl-sulphonium salt) could be an intermediate in transmethylation. This would necessitate the methylation of methionine, followed by the sulphonium salt acting as a methyl donor, in a cyclic process, as shown in Fig. B.

Bennett (1941) showed that this substance, methionine-methyl-sulphonium-chloride, could act as a methyl donor in rat growth experiments. One can criticize the above theory on the grounds that methionine-methyl-sulphonium chloride has not been detected in tissues, but neither has homocysteine, so that a similar criticism can be levelled at any theory which involves the latter substance. Of course/

course, if either of the substances, homocysteine or methionine-methyl-sulphonium chloride, were acting in a catalytic capacity in a chain of reactions, then its concentration might be so low that it would escape detection.

Bennett (1941) made the pertinent observation that whereas methionine sulphoxide would support the normal growth of young rats, methionine sulphone would not. The primary oxidation step in the conversion of methionine to the sulphoxide might therefore be an essential reaction in the 'labilising' of the methyl radical, but of course this hypothesis would appear to be incompatible with the methionine-methyl-sulphonium salt theory which was previously advanced.

In short, the following substances - choline, betaine, methionine, methionine sulphoxide, methionine-methyl-sulphonium salt, dimethyl-β-propiothetin and dimethylthetin (Maw et al., 1948) are all known to be potential labile methyl donors. Whether the mechanism for the transfer of a methyl group to (say) glycocyamine, follows several independent pathways or involves certain of these substances in a chain of reactions is still not known.

The/

The physiological action of 'Labile' methyl groups

The labile methyl group appears to be required as a dietary factor to support the optimum growth of the young white rat. This accessory dietary factor can also cure or prevent the fatty liver condition which follows pancreatectomy. Closely associated with this lipotropic action of the labile methyl group is the renal anti-haemorrhagic action of this radical. Animals maintained on a diet in which all labile methyl groups have been withheld, develop haemorrhagic kidneys; now all methyl donors (established by the rat growth technique) are effective in preventing both the fatty liver and the renal lesion syndromes. On the other hand, certain compounds devoid of labile methyl groups, for example triethylcholine, are effective as both lipotropic and anti-haemorrhagic agents. It does seem from the very nature of the substances which alleviate these two conditions, that the specific grouping required is a strongly polar residue with an alcoholic group, which can be incorporated by an ester linkage into a phosphatide, in order to facilitate fat transport. This requirement/

requirement is satisfied by quadrivalent nitrogen, with one substituent a hydroxylated alkyl radical, as in choline or triethylcholine, but as the latter substance has not been detected in nature, methyl groups probably exert a lipotropic action through the synthesis of choline. These aspects of methyl group metabolism associated with fatty livers and renal lesions were both reviewed in 1941 by McHenry and Griffith respectively.

As a result of the work on rat growth, liver fat and haemorrhagic kidneys reviewed in this section, the theory was advanced that the body could not synthesize the methyl group, and this radical was accordingly raised to the status of an 'essential dietary factor'. The possibility that methyl groups are being synthesized in the body at a rate insufficient to satisfy the requirements of catabolism is implicit in the results of Bennett (1944) obtained in rat growth experiments. Bennett found that all the animals in her rat colony could grow at a sub-optimal rate on a diet free from labile methyl groups. This excellent work which pointed to the significant biosynthesis of methyl groups in mammals was viewed with reserve by many workers who/

who preferred to accept the simpler 'vitamin aspect' of the methyl group problem. The critics explained the anomalous results as due to the absorption of methylated compounds produced by the symbiotic micro-organisms in the animals' alimentary tract.

Outline of the Present Problem

The work which has been reviewed leads to three conclusions.

1. Little is known of the origin of the methyl radical, although much evidence supports the idea that this grouping cannot be rapidly synthesized by mammals. It is important, however, to note in this connection the recent isotope work (Sakami, 1949), (Sakami, 1950; Arnstein, 1950) which links the methyl group with glycine, serine, and other non-methyl compounds.
2. Much is known about transmethylation reactions in which the methyl group is transferred intact from a donor to an acceptor molecule. In fact, this work is reaching the stage at which the action of enzymes such as choline oxidase and transmethylase is rightly receiving considerable attention. It is not, however/

however, known which is in practice the more important of the two established methyl donors, choline (or betaine) and methionine, or indeed, to what extent transmethylation is a major pathway for the metabolism of either compound.

3. Although the apparent necessity for continuous supply implies constant loss or destruction of methyl groups, their ultimate fate remains quite unknown. Although methyl groups yield respiratory carbon dioxide (Mackenzie, 1949, 1950), the mechanism by which this is produced from the 'one carbon fragment' is completely obscure.

It was felt that suitable work on the metabolism of methyl groups in a closed physiological system might provide information of some value in filling in the gaps in present knowledge, more especially those to which attention is directed in paragraph 2 above. Consideration of possible systems led to the selection of the embryonated hen egg, in which the chick embryo develops during incubation at the expense of a ready-made/

made culture medium, which has long been known to be rich in lecithin - and therefore in choline. It has been found in the present work to contain methionine in comparable amounts. It is true that a system comprising a micro-organism and a synthetic culture medium would have had a less variable composition at the outset of each experiment, but it was felt that this defect could be compensated by the analysis of a sufficient number of eggs at each stage of incubation. The advantages for the purposes in mind of making observations on a naturally occurring system appeared to outweigh all such minor disadvantages. Caution however, should be exercised in the application of the results, since embryonic tissue behaves differently from normal tissue in many respects, and appears to bear many resemblances to neoplastic tissues.

Experiments were accordingly developed along the following lines:

1. Estimations: Quantitative determination of methyl donors and methylated compounds such as choline, acetylcholine, methionine, creatine and creatinine/

creatinine in both the embryo and its 'culture medium' at various stages of development. From the results it would be possible to construct a 'balance sheet' of the labile methyl groups in the complete closed system at any given stage of incubation. Such a technique would show not only the progressive transference of methyl groups from the 'medium' to the embryo, but might also be expected to provide general information about the rates at which the different compounds were metabolised. If necessary, the allantoic fluid could be analysed separately from both the embryo and the 'medium' for excretory products such as creatine and creatinine.

Determinations of acetylcholine were made. The quantities found were minute, and did not affect any of the points at issue.

2. Studies on the bases present in the system, particularly in the embryonic tissue:

It was felt in view of the work of Dubnoff (1949) and Muntz (1950) which has already been reviewed, that a knowledge of the presence or absence of betaine, ethanolamine, monomethylaminoethanol, dimethylamino-ethanol/

ethanol, sarcosine and dimethylglycine would be of interest in connection with the metabolism of choline. In this part of the work only qualitative experiments were undertaken. Even where quantitative methods are available, it was thought that the results of their application to intermediate compounds in an actively working system would be unlikely to justify the work.

3. Studies on enzyme systems of possible importance: Studies were undertaken on enzyme systems such as choline oxidase, transmethylase and other enzymes thought to be directly involved in the metabolism of methyl groups. It seemed possible that a knowledge of the activity of these enzymes in the chick embryo might provide useful information about the reactions taking place. It was hoped that it might be possible to extend these studies to the intermediary metabolism of 'one carbon compounds'.

Before proceeding to the detailed account of the experimental work, it will be necessary to refer briefly to certain published work bearing directly on the quantitative determinations. This is dealt with in the next section.

Review/

Review of Previous Work on the Chick Embryo relevant to this problem.

Many observations have been made of chemical changes occurring during the development of the chick embryo, and much of the older literature is collected in the comprehensive monograph on 'Chemical Embryology' by Needham (1931) and at a later date the same author (1942) reviews other aspects of this subject in his treatise 'Biochemistry and Morphogenesis'.

Choline Metabolism

The choline content of the hen's egg during incubation was studied by Sharpe (1924) and Okada (1922). Sharpe, using the enneaiodide procedure for the estimation of choline, found that this substance decreased from about 400 mg./ 100 g. at the commencement to 220 mg./ 100 g. at the end of incubation. As this work was based on only four points during incubation (0, 7, 14 and 21 days), it is open to a great deal of criticism.

The results obtained by Okada were similar, but whereas Sharpe found 10% of the total choline at/

at the end of incubation to be 'free' (that is, not in combination as lecithin), Okada found the free choline to amount to 80% of the total present at the end of incubation.

Nakamura (1928), using a gravimetric method for the estimation of choline (platinum salt), found only 50% of the choline as recorded by Sharpe. The important feature of Nakamura's results is that he found that even at the end of incubation, the free choline never attains a value higher than 3 mg. per egg, which is less than 1% of the total choline present at the onset of incubation. Okada on the other hand, had found about 80 mg. free choline per egg at the termination of incubation. Although inadequate experimental methods led to such discrepancies between the different sets of results, all the workers were in agreement on two points: (a) that all the choline present in the unincubated egg was bound as lecithin and (b) that the total choline decreased appreciably during incubation. As all the choline in the unincubated egg was bound as phospholipin, the metabolism of phosphorus must be linked quite closely with the metabolism of choline.

Alterations/

Alterations in the phosphorus distribution in the developing chick embryo were studied by Plimmer and Scott (1908). These workers found that the 'ether soluble phosphorus' diminished from 65% of the total phosphorus at the onset of incubation to 20% of the total at the end of incubation. Using the figures of Plimmer and Scott and assuming that practically all the choline in the unincubated egg was present as lecithin, it is possible to calculate the amount of choline in the system. Provided the ratio of choline-containing phospholipins to total phospholipins did not alter during incubation, there would be a disappearance of almost 70% of the original choline bound as lecithin. This would imply either disappearance of choline from the system or its accumulation in some form other than in chemical combination as phospholipin.

The metabolism of choline during the development of the chick embryo thus required elucidation: if this closed system did contain much free choline, then it would be very different from most tissues, in which the free choline concentration is very low.

Creatine/

Creatine and Creatinine Metabolism

Mellanby in 1907 failed to find any creatinine in the unincubated egg, while Salkowski (1911) found traces of this compound. The important point is, however, that the creatine or creatinine content of the unincubated egg must be very small. Mellanby estimated the creatine and creatinine content of the developing chick embryo from the 14th day until hatching; at the latter point he found approximately 22 mg. creatine to be present in the hatching chick. Sendju (1927) in some detailed experiments, found the creatinine per embryo to be very small (less than 1 mg.); he also carried out creatine and creatinine estimations on the allantoic fluid.

Methionine Metabolism

Suitable analytical techniques for the accurate estimation of this amino acid only became available quite recently, and no estimations of methionine on the chick embryo are to be found in the literature.

Csonka et al. (1947) performed a number of analyses on unincubated eggs and found between 150-190 mg. methionine per egg.

Acetyl/

Acetyl Choline Metabolism

Szepsenwol and Caretti (1942) estimated an 'acetyl-choline-like' substance in parts of the chick embryo, but they did not make any overall measurements of this substance. Malcolm (1950) estimated acetyl-choline in sheep embryos and found about 3-4 microgrammes acetyl choline per gram of embryo dry weight.

It will thus be seen that although experiments to estimate compounds containing labile methyl groups in the chick embryo have been carried out over a period of fifty years, much remains to be done. By using the newer, more specific, methods of estimation of choline, etc., it was felt that data could be collected which might throw light on the quantitative significance of certain trans-methylation reactions.

EXPERIMENTAL.

CHEMICAL DETERMINATION OF METHYLATED COMPOUNDS.

Preparation of material.

Eggs from Brown Leghorn hens fed on a standard breeding diet were supplied by the Poultry Research Centre, Edinburgh. The experiments were conducted throughout the year except during November and December when the fertility of the eggs is low. In order to minimise the number of analyses the weight of the eggs at the onset of incubation was limited to 55±5g. The eggs were electrically incubated under normal conditions of temperature and humidity. After six days' incubation, the fertility was tested and all infertile eggs discarded. At the end of the desired incubation period the eggs were removed, weighed and frozen for ten minutes in a mixture of acetone and solid carbon dioxide. The frozen eggs were left at room temperature for two minutes and the shells quickly stripped off and weighed. The weight of the egg contents was found by difference. The embryo was dissected out and weighed, and the weight of the 'Remainder' again found by difference. The fraction designated/

designated 'Remainder' consisted of the residual yolk, white, allantoic fluid, amniotic fluid, and the membranes. The partition of the egg in preparation for analysis is illustrated in Figure 1 below.

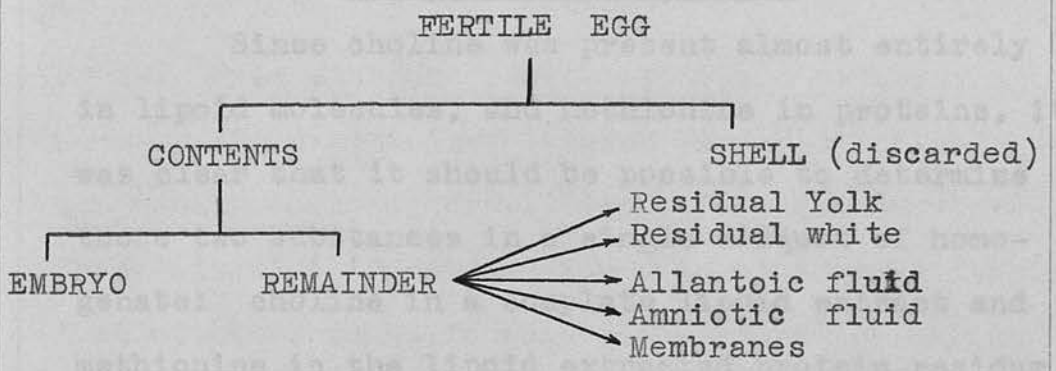


Figure 1.

The 'Embryo' and the 'Remainder' were homogenised with measured volumes of water in separate ice-chilled glass homogenisers(Potter& Elvehjem,1936) The prepared suspensions were stored in the refrigerator until required. The analyses were usually commenced within a few hours of the preparation of the homogenates.

Sampling for Analysis.

The most convenient method of removing an aliquot from the homogenate was by pipette. As the original/

original measurements on 'Embryo' and 'Remainder' were by weight, it was theoretically necessary to apply a density correction. In actual fact the density of the homogenates was sufficiently close to unity for this correction to be neglected.

Preparation of Extracts

Since choline was present almost entirely in lipoid molecules, and methionine in proteins, it was clear that it should be possible to determine these two substances in a single aliquot of homogenate: choline in a complete lipoid extract and methionine in the lipoid extracted protein residue. As creatine and creatinine can only be determined in an aqueous protein-free extract it would be necessary to take another aliquot of homogenate for these estimations.

Lipoid Extraction (for choline)

A measured volume (usually 2 ml.) of homogenate was pipetted into 20 ml. chloroform-methanol (1:1)in a 6" x 1" boiling tube, and the mixture refluxed for ten minutes, cooled and centrifuged. The supernatant fluid was decanted off and the extraction of the residue repeated with three further portions of 20 ml. of the solvent. The extracts were pooled and made up to 100 ml. and this solution used for the determination of choline.

The/

The efficiency of this process was tested by experiments as reported in Appendix I.

Protein Residue (for Methionine)

The residue from the lipoid extraction was washed once with ether and dried. This residue was used for the estimation of methionine.

Protein-free Extract (for Creatine and Creatinine)

The protein precipitant favoured for this purpose by most workers is tungstic acid, and in this investigation it was found to be quite suitable. A solution of tungstic acid was prepared by mixing equal volumes of N sulphuric acid, and 10% sodium tungstate. Protein-free extracts of homogenates of 'Embryo' and 'Remainder' were prepared as follows:

(a) Embryo: An aliquot of the homogenate (2.5 ml.) was added to 42.5 ml. water and this suspension was treated with 15 ml. tungstic acid, prepared as above. The solution was allowed to stand in ice for fifteen minutes and filtered through a dry No. 42 filter paper.

(b) Remainder: An aliquot of the homogenate (10 ml.) was added to 20 ml. water, and to this suspension 15 ml. tungstic acid, the resultant mixture being treated exactly as before.

CHEMICAL METHODS

(a) CHOLINE

The method selected for the determination of choline was based on the hydrolysis of the phospholipins with barium hydroxide and precipitation as choline reineckate $(CH_3)_3\overset{+}{N}CH_2CH_2OH\left[(NH_3)_2Cr(CNS)_4\right]^-$. The amount of the washed precipitate was either estimated volumetrically by the procedure of Ramsay and Stewart (1941) or photometrically after solution in acetone (Beattie, 1937). The two methods gave identical results, but the volumetric method proved to be unnecessarily delicate and undesirably tedious. The great majority of the analyses were therefore done photometrically as described below.

Reagents: Saturated aqueous barium hydroxide.

1.0 N and 0.1 N sulphuric acid.

Saturated aqueous solution of ammonium reineckate, freshly prepared.

0.1 N hydrochloric acid.

Acetone.

Procedure/

Procedure:

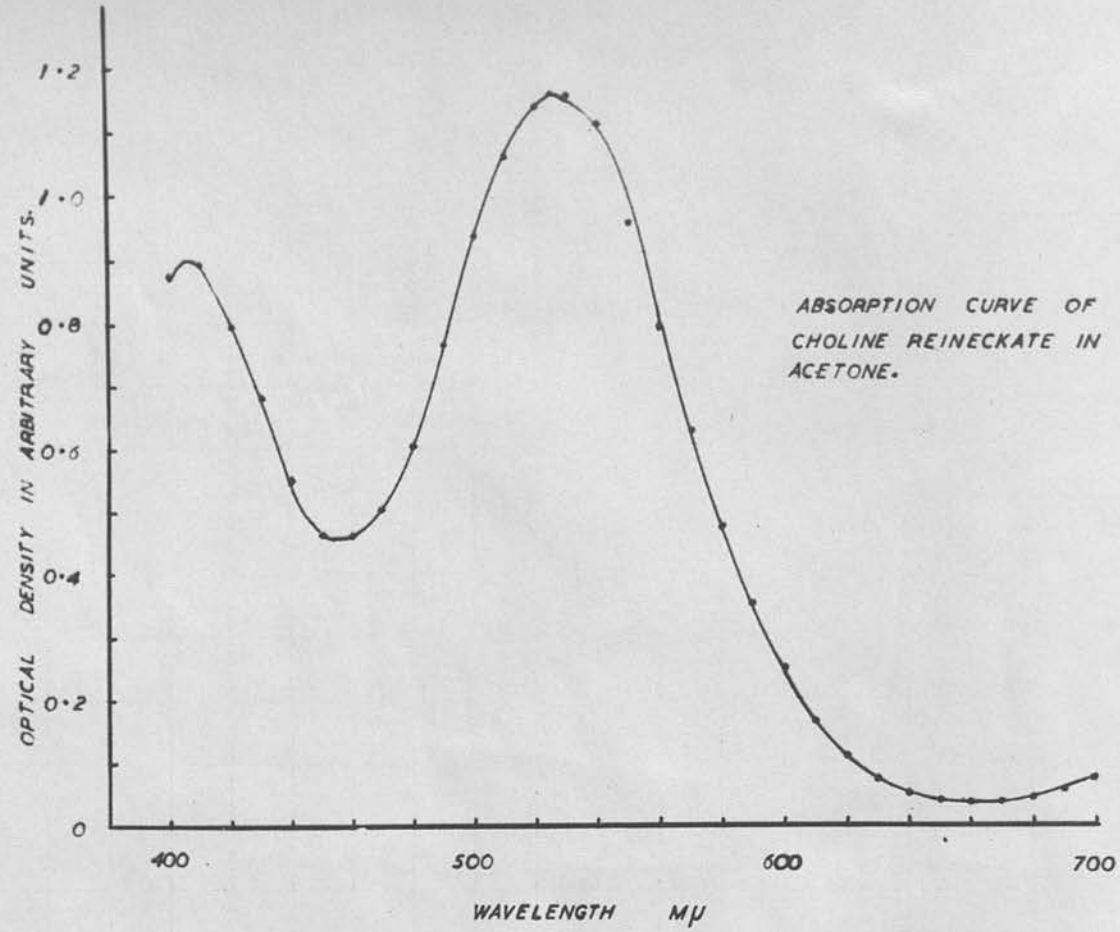
1. An aliquot of the lipoid extract (5-10 ml.) was taken to dryness in a 15 ml. graduated tube, and hydrolysed with 3 ml. of a saturated aqueous solution of barium hydroxide for two hours at 100°C.

2. While still in the water-bath, the hydrol-:ysate was treated with 1.0 N sulphuric acid, and then 0.1 N sulphuric acid until the pH was 3-4, using methyl red as the indicator. This solution was made up to 15 ml. and filtered (No. 42 paper).

3. A 10 ml. aliquot of this filtrate was delivered into a spectrophotometer tube graduated at 8 ml., and treated with 2 ml. of a freshly prepared saturated aqueous solution of ammonium reineckate.

4. The choline reineckate precipitate was left in the refrigerator for at least two hours, and then centrifuged at 3000 r.p.m. for twelve min-:utes. The supernatant liquid was then dis-:carded and the precipitate was washed twice with 7-10 ml. ice cold 0.1 N hydrochloric acid, and twice with 7-10 ml. ice cold distilled water, centrifuging each time as before.

5. Finally/


1·2
1·0
0·8
0·6
0·4
0·2
0
OPTICAL DENSITY IN ARBITRARY UNITS.
400
500
600
700
WAVELENGTH Mμ
ABSORPTION CURVE OF CHOLINE REINECKATE IN ACETONE.

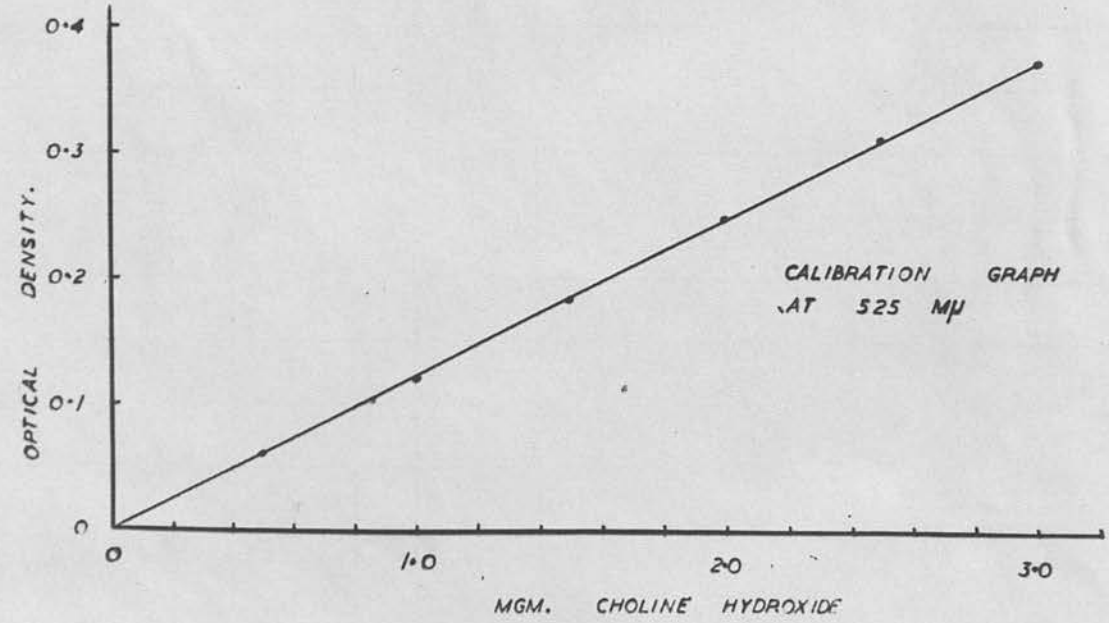

0·4
0·3
0·2
0·1
0
OPTICAL DENSITY.
0
1·0
2·0
3·0
MGM. CHOLINE HYDROXIDE
CALIBRATION GRAPH AT 525 Mμ

FIGURE 2.

5. Finally the precipitate was dissolved in 8 ml. acetone and read against an acetone blank in a diffraction grating spectrophotometer at wavelength 525 mμ .

6. Identical calibration graphs were obtained by the use of

(i) standard solutions of choline reineckate in acetone, and,

(ii) standard solutions of choline chloride (checked by micro-Kjeldahl analysis)

carried through the procedure from stage 3 on. Figure 2 shows the absorption spectrum of the choline reineckate solution and a typical calibration graph.

Experiments to ascertain the reliability of this procedure are reported in Appendix I.

(b) METHIONINE

The method used in the determination of methionine was modified slightly from the McCarthy and Sullivan (1941) procedure. This method required hydrolysis of the protein with hydrochloric acid followed by neutralisation, and photometric estimation of methionine utilising the colour reaction which it gives with nitroprusside in acid solution.

Reagents/

Reagents: 4 N hydrochloric acid

5 N sodium hydroxide

2 N acetic acid

1% glycine

14 N sodium hydroxide

10% sodium nitroprusside solution, freshly prepared.

Mixed acid (90 ml. concentrated hydro-:chloric acid plus 10 ml. 85% phosphoric acid)

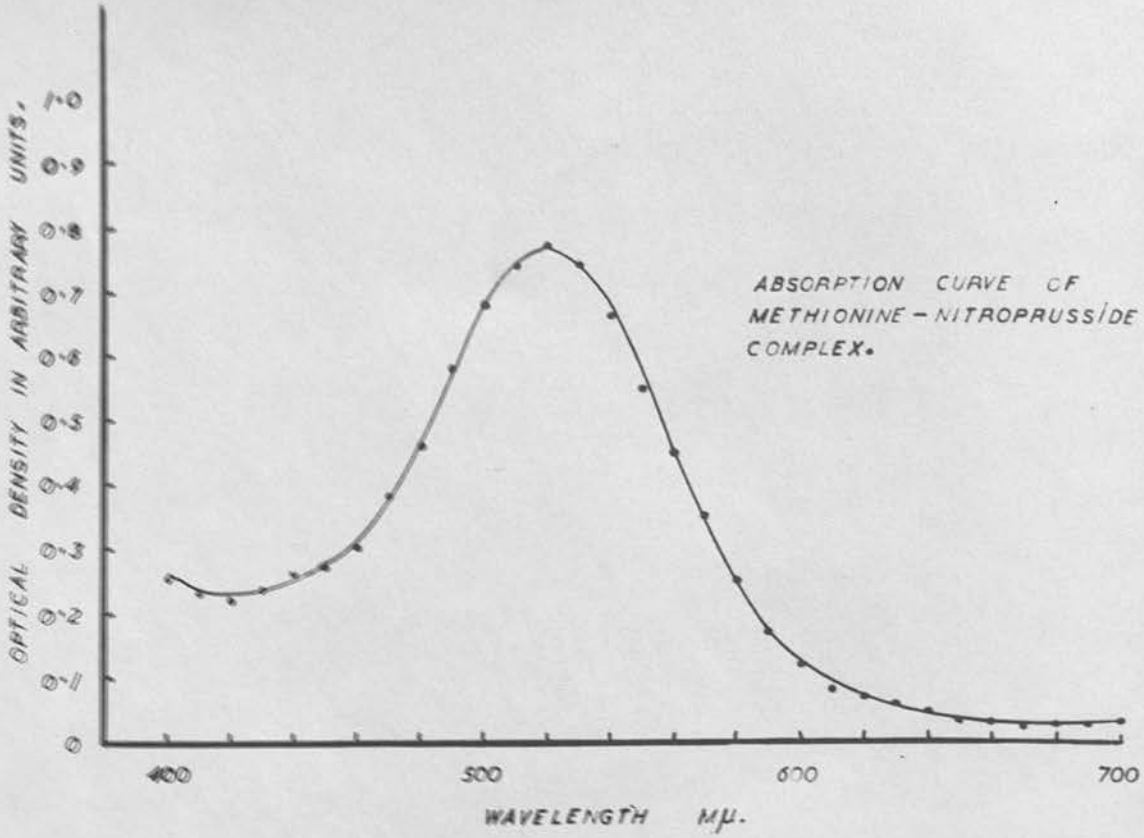
Procedure:

1. The residue from the lipoid extraction was washed once with ether and dried. This residue (in the same tube as used in the lipoid extrac-:tion) was refluxed with 6 ml. 4 N hydrochloric acid for six hours.

2. The hydrolysate was diluted with 5 ml. water, treated with 100 mg. "Norit", and heated for two hours on a boiling water bath. This solution was filtered through a micro-Hirsch funnel, washing the residue with warm 0.1 N hydrochloric acid.

3. The combined filtrates were quantitatively transferred to a 25 ml. graduated flask which was chilled in ice. This acidic hydrolysate was

carefully/


OPTICAL DENSITY IN ARBITRARY UNITS.
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
400
500
600
700
WAVELENGTH mμ.
ABSORPTION CURVE OF METHIONINE – NITROPRUSSIDE COMPLEX.

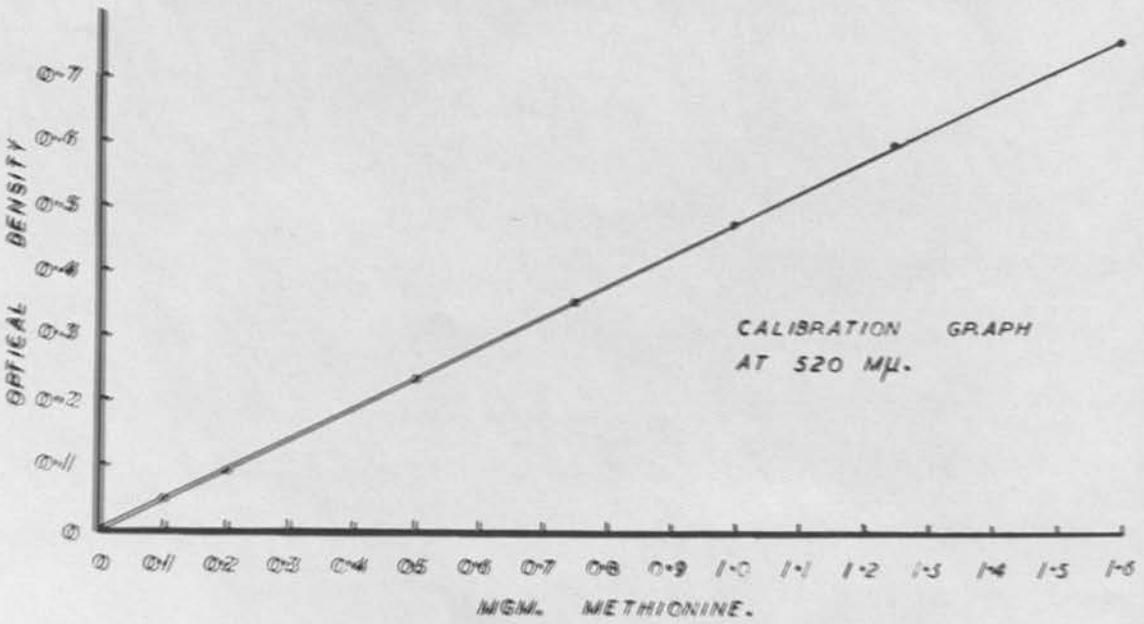

OPTICAL DENSITY
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·1
1·2
1·3
1·4
1·5
1·6
MGM. METHIONINE.
CALIBRATION GRAPH AT 520 mμ.

FIGURE 3.

carefully treated dropwise with 5 N sodium hydroxide until just alkaline, and then 2 N acetic acid added until the pH was about 5.

4. The flask was removed from the ice bath and allowed to attain room temperature and made up to volume with distilled water.

5. Aliquots of this solution (2-5 ml.) were transferred to 15 ml. graduated tubes, and 1 ml. 1% glycine, 1 ml. 14.0 N sodium hydroxide and 0.3 ml. 10% sodium nitroprusside added to the hydrolysate.

6. This mixture was heated at 40°C for 10 minutes, chilled in ice for 2 minutes, and 5 ml. "mixed acid" run into the solution from a burette.

7. The red solution was made up to the mark with distilled water, well shaken, and read at once against a reagent blank on the diffraction grating spectrophotometer at 520 mμ.

8. The absorption curve of the methionine nitroprusside complex, and the calibration graph, are shown in Figure 3. The calibration graph was constructed from pure methionine and the standards were prepared with sodium chloride and/

and sodium acetate solutions in the same proportions as those in which they were present in the hydrolysate (see Appendix I), because these salts were found to depress slightly the methionine colour reaction. This point escaped the notice of the original authors (McCarthy and Sullivan, 1941).

Experiments to clarify the following points are described in Appendix I:

(i) Conditions of hydrolysis.

(ii) Efficiency and reliability of the "Norit" decolourising process.

(iii) The effect of sodium chloride on the colour reaction.

(iv) Possible interference with the colour reaction by other amino acids.

(c) CREATINE AND CREATININE

Creatine and creatinine were determined by the well known Jaffe (1886) reaction using alkaline picrate in the modification of Peters (1942). Although this reaction is not specific for creatinine, control experiments (see section on "Bases occurring in the System") in which extracts of the materials analysed/

analysed were subjected to paper chromatography, showed the absence of all the interfering substances listed by Hunter (1928). This finding made it unnecessary to have recourse to the enzymic refine-:ment of Miller and Dubos (1937, a). Similar conclusions for a number of other tissues were reached by both Miller and Dubos (1937, b, c) and Beard (1943).

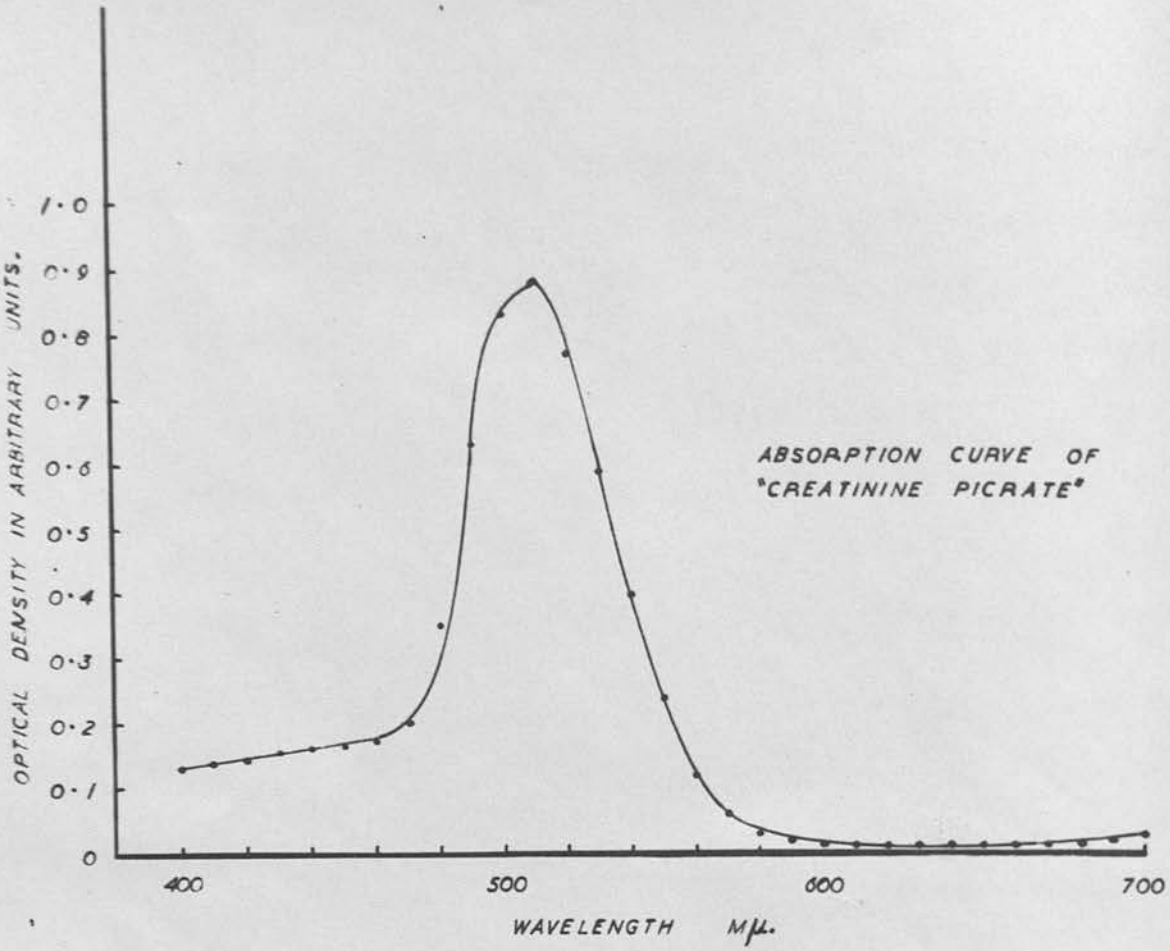
Reagents: 1.1% aqueous picric acid solution
10% sodium hydroxide

Procedure for Creatinine:

An aliquot of the protein free tungstic acid filtrate was transferred to a 20 ml. graduated test tube and treated with 5 ml. of alkaline picrate solution, prepared by mixing 1 part 10% sodium hydroxide with 4 parts 1.1% picric acid solution. The yellow-red solution was made up to volume and allowed to stand for 20 minutes before being read against a reagent blank in the spectrophotometer at wavelength 510 mμ.

Procedure for Creatine:

An aliquot of the protein free tungstic acid filtrate was transferred to a 20 ml. graduated tube and N hydrochloric acid added if necessary, to bring/


OPTICAL DENSITY IN ARBITRARY UNITS.
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
400
500
600
700
WAVELENGTH Mμ.
ABSORPTION CURVE OF
"CREATININE PICRATE"

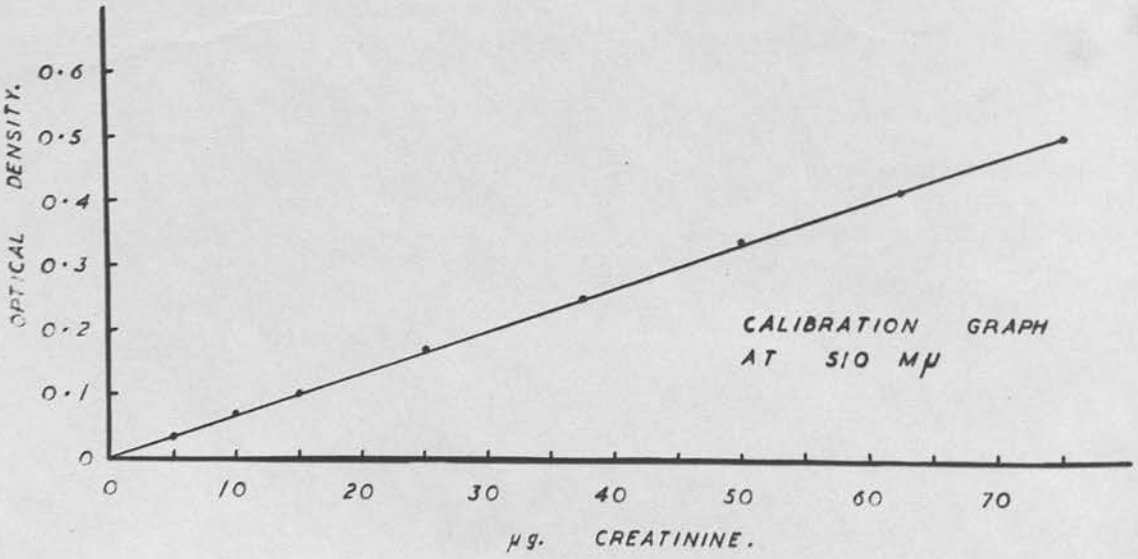

OPTICAL DENSITY.
0·6
0·5
0·4
0·3
0·2
0·1
0
0
10
20
30
40
50
60
70
μg. CREATININE.
CALIBRATION GRAPH
AT 510 Mμ

FIGURE 4.

bring the pH of the solution to unity.

2. The tubes were capped with lead foil and autoclaved for 30 minutes at 120°C.

3. The tubes were allowed to cool and were then treated with alkaline picrate solution exactly as before, estimating therefore the total creatinine which is the preformed creatinine plus the creatine estimated as creatinine.

An absorption curve of "creatinine picrate" was constructed and showed a maximum absorption at wavelength 520 mμ . A calibration graph was constructed using pure creatinine as standard, in 20 ml. graduated tubes, and using the same volume of alkaline picrate as above. Both these graphs are reproduced in Figure 4.

Before any determinations on the homogenates were attempted, and immediately the calibration graph was found to be reproducible, the following essential check experiments were conducted.

(i) The rate of colour development.

(ii) The optimum conditions of pH, time and temper-:ature for the quantitative conversion of creatine to creatinine.

(iii) Recovery experiments on creatine and creatin-:ine.

These experiments are reported in Appendix I.

ANALYTICAL RESULTS.

Distribution of methylated compounds during the development of the chick embryo.

The experimental techniques described in the preceding section were applied to 16 unincubated eggs and 201 eggs incubated for periods between 6 and 21 days. The quantities of choline, methionine, creatine, and creatinine were determined in both the embryo and the 'remainder' at approximately two day intervals of incubation. The 'age' of the embryo was estimated from its weight, employing the chick embryo growth curve constructed by Byerly (1932).

As there was considerable variation from egg to egg, 6 - 16 eggs were analysed at each stage of incubation. The results are tabulated in detail in Tables A, B, and C, (see Appendix III). The creatinine figures have been omitted because they were negligibly low. Certain broad trends which emerge are clearly seen in Figures 5,6, and 7.

Comparison of results obtained on unincubated eggs with those of other workers.

The choline content (expressed as choline hydroxide) of 16 unincubated eggs averaged 181 mg. per egg/

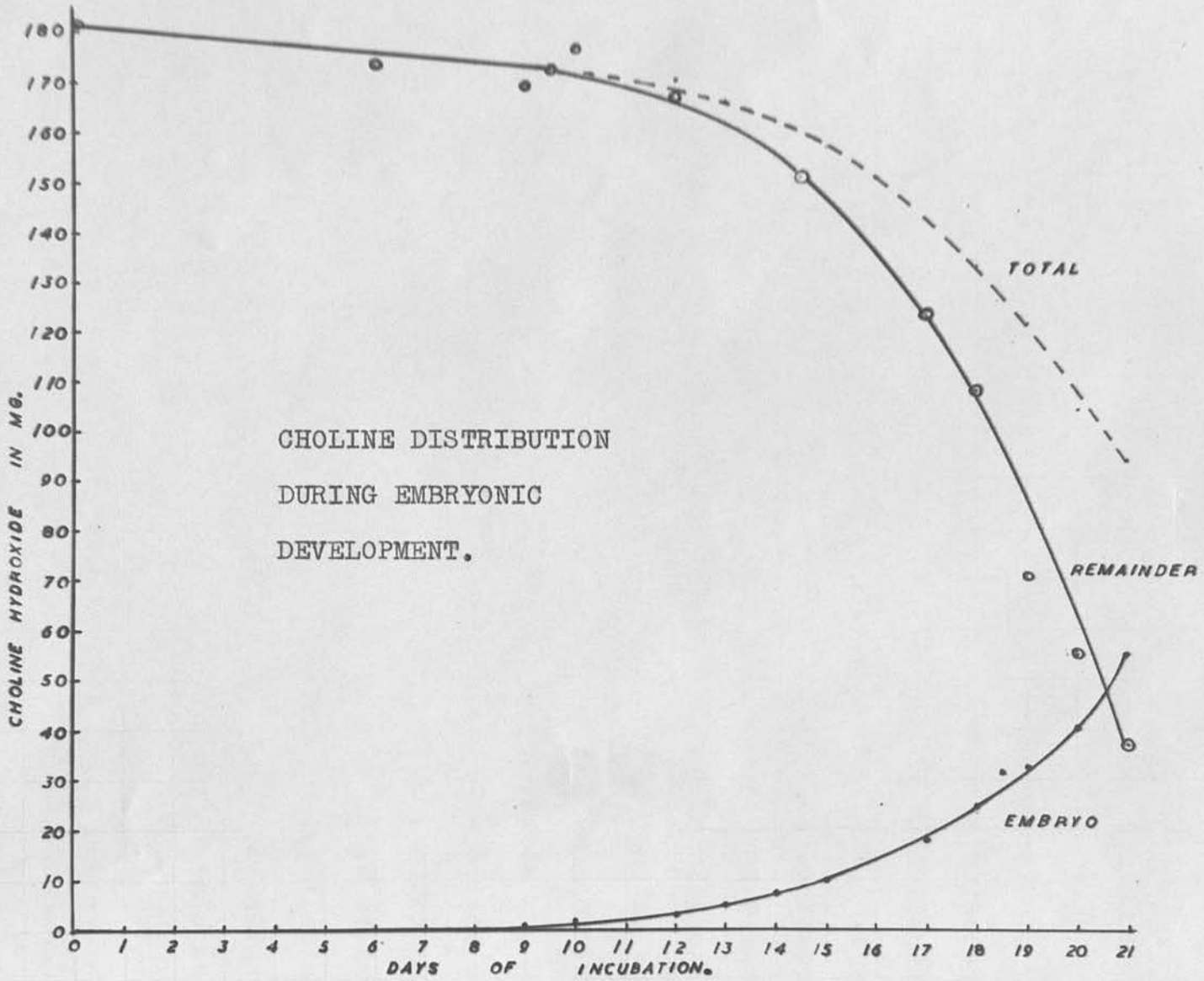

CHOLINE DISTRIBUTION
DURING EMBRYONIC
DEVELOPMENT.
TOTAL
REMAINDER
EMBRYO
CHOLINE HYDROXIDE IN MG.
DAYS OF INCUBATION.
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

FIGURE 5.

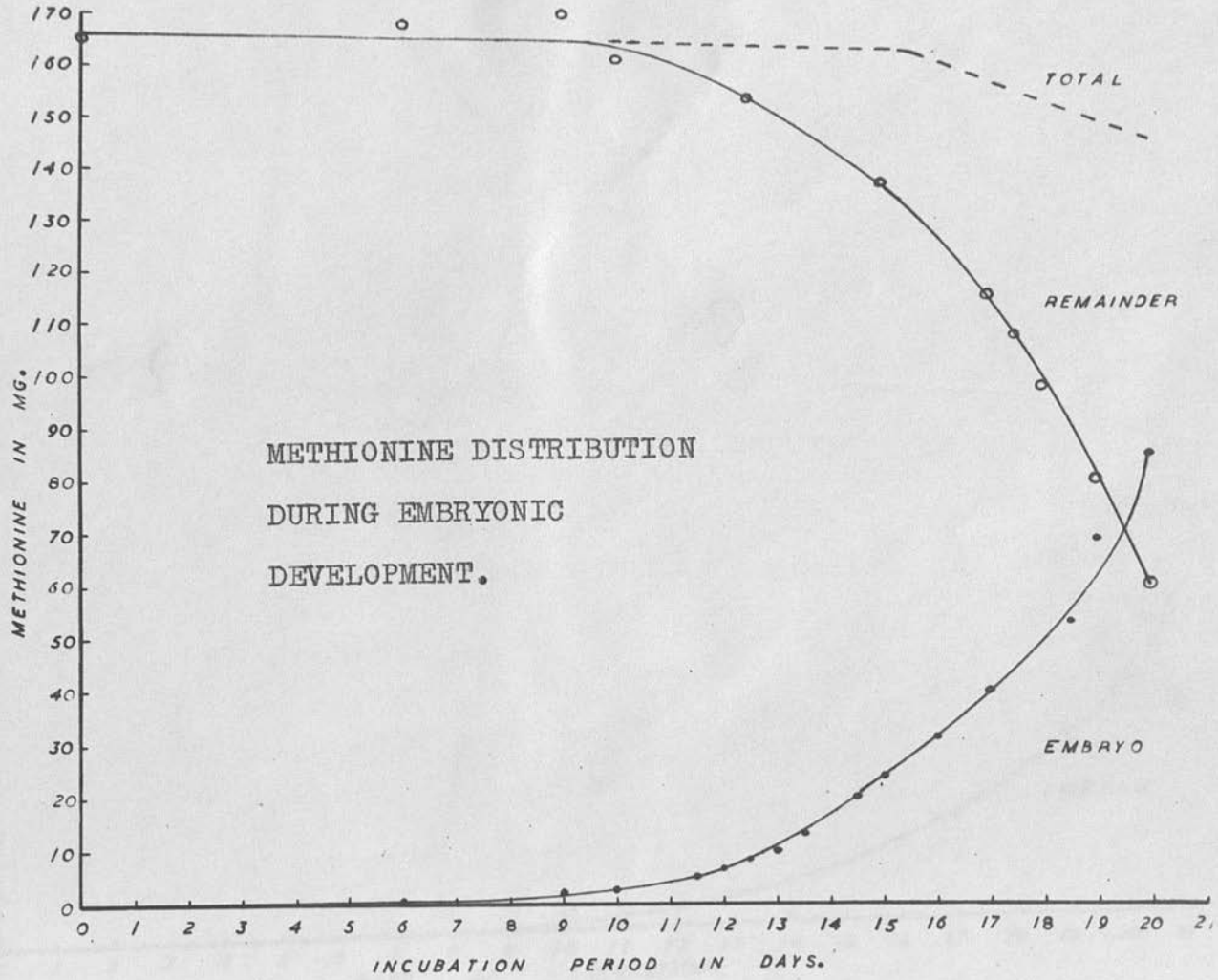

METHIONINE DISTRIBUTION
DURING EMBRYONIC
DEVELOPMENT.
TOTAL
REMAINDER
EMBRYO
METHIONINE IN MG.
INCUBATION PERIOD IN DAYS.
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

FIGURE 6.

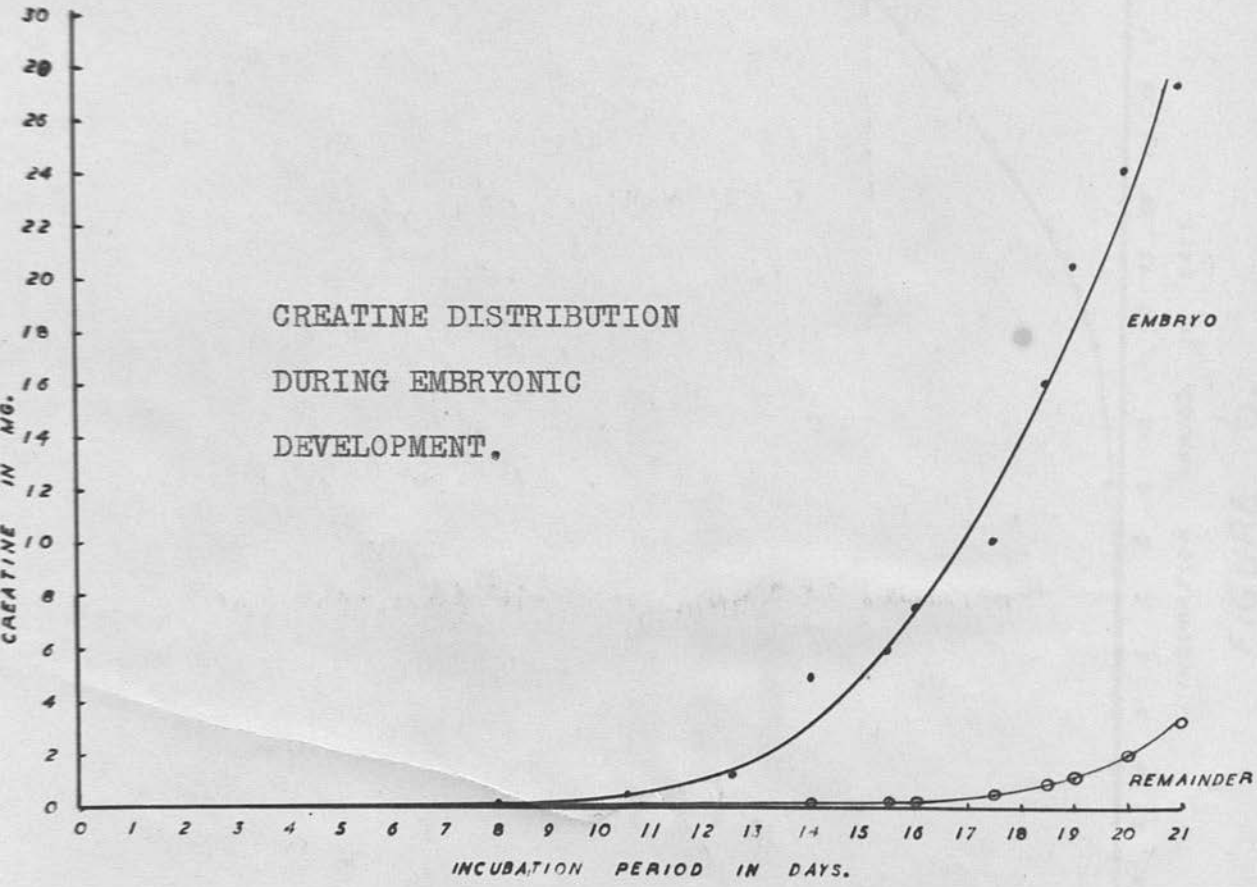

CREATINE DISTRIBUTION
DURING EMBRYONIC
DEVELOPMENT.
EMBRYO
REMAINDER
CREATINE IN MG.
INCUBATION PERIOD IN DAYS.
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

FIGURE 7.

egg. This figure is remarkably close to that of Horowitz and Beadle (1943) who made their determin-:ations by microbiological assay. The methionine content of 14 unincubated eggs averaged 165 mg. per egg. This figure is satisfactorily similar to that of Csonka and Denton (1947) who used almost the same analytical method. The virtual absence of creatine and creatinine from the unincubated egg (Mellanby,1907) was confirmed.

Results obtained on incubated eggs.

In the embryo, all the chemical constituents increased in the expected exponential manner to a maximum value at the time of hatching of 56 mg. choline, 86 mg. methionine, and 27 mg. creatine. At the same time the quantities of both choline and methionine in the 'remainder' decreased; the rate of this decrease being very marked towards the end of incubation. The creatine content of the 'remainder' increased dur-:ing incubation, but this is entirely attributable to the excretion of creatine by the embryo into the allantoic fluid which is included in the 'remainder'.

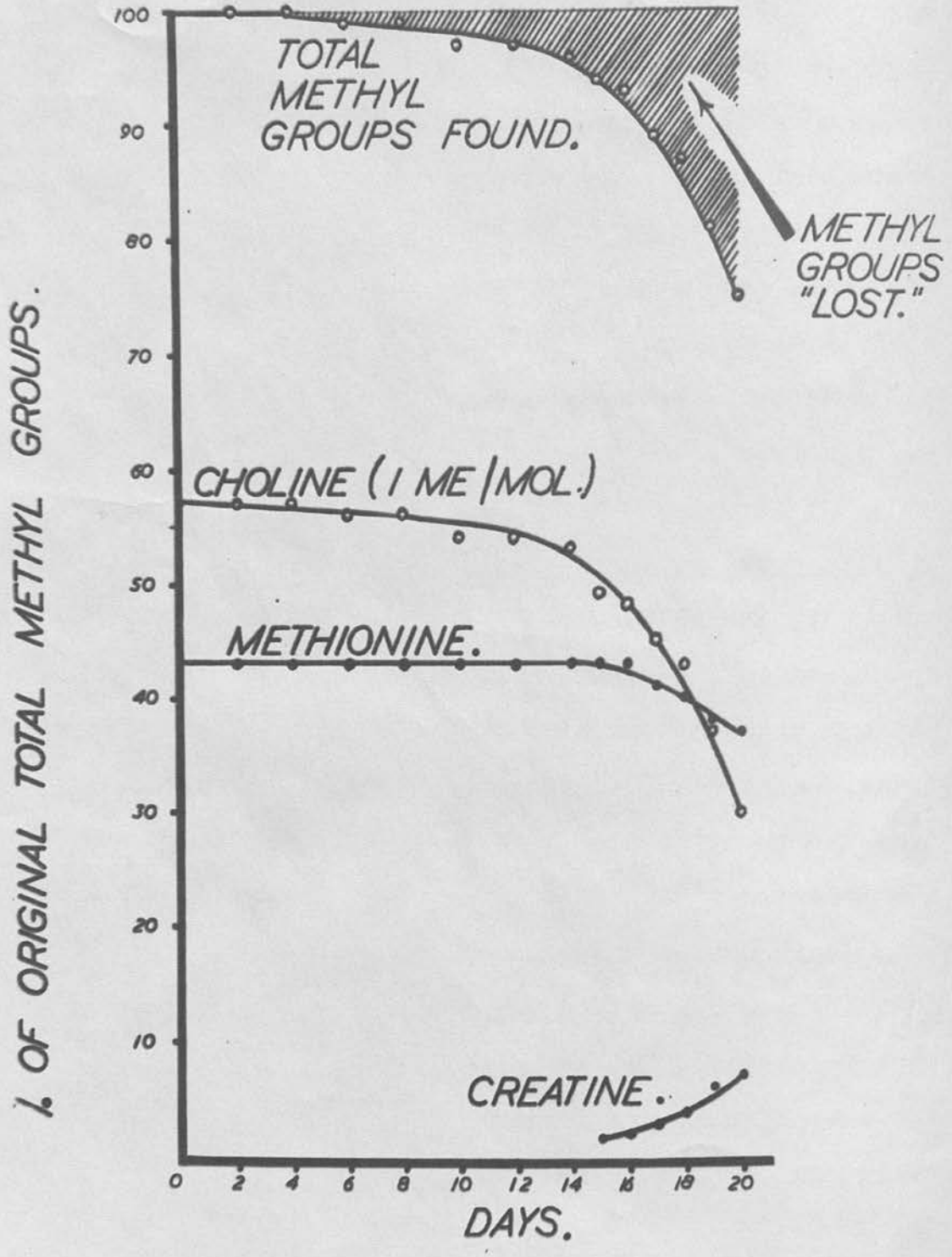

% OF ORIGINAL TOTAL METHYL GROUPS.
100
90
80
70
60
50
40
30
20
10
TOTAL METHYL GROUPS FOUND.
METHYL GROUPS "LOST."
CHOLINE (1 ME/MOL.)
METHIONINE.
CREATINE.
0
2
4
6
8
10
12
14
16
18
20
DAYS.

FIGURE 8

Methyl Group Balance Sheet.

It is believed that choline, creatine, and methionine account for a large proportion of the total 'labile' methyl groups in the developing egg. Although the methyl group in creatine is not itself 'labile' (duVigneaud 1945, 1950) it is derived from the pool of 'labile' methyl groups, and must therefore be included in the total. Both creatinine and acetylcholine have been shown by analysis to be present in only minute quantities, and it will be demonstrated that methylated bases such as, sarcosine, anserine, betaine and others are either absent, or present only in traces. It is suggested by Sprinson et al (1950) that the methyl group of thymine may be derived from the 'labile' methyl group pool, but calculation from the deoxyribonucleic acid figures of Novikoff and Potter,(1948) or Sutherland, (1950, unpublished) shows that the quantity involved is again extremely small.

These considerations make it highly probable that no serious error would arise in the compilation of a methyl group 'balance sheet' from the choline, methionine, and creatine figures. This balance sheet is drawn up in Figure 8, which shows the total amount/

amount of 'labile' methyl groups accounted for by each of these three substances in the system as a whole (embryo and 'remainder') during the course of incubation. The results are expressed as a percentage of the initial quantity of 'labile' methyl groups present in the system at the onset of incub-:ation. For the purpose of this graph, choline is assumed to have only one 'labile' methyl group per molecule, (du Vigneaud et.al., 1942)

It will be observed from Figure 8, that the methionine content of the system remains constant until about the 17th day of incubation. Creatine synthesis proceeds constantly, presumably at the expense of choline because the total amount of this substance decreases by 50%, more than enough to account for all the methyl groups associated with the creatine synthesised. This suggests that choline is the chief ultimate methyl donor in this system at least during the major part of embryonic development, a fact which lends additional importance to the study of methylated bases reported in the next section.

This 'Balance Sheet' of 'labile' methyl groups shows/

shows that during embryonic development, the total methyl groups accounted for as choline, methionine, and creatine, decreases by approximately 25%. The fact that this substantial proportion of the 'labile' methyl groups cannot be accounted for by known trans-methylation reactions will be dealt with in the section entitled 'Discussion'.

EXPERIMENTAL.

ACETYLCHOLINE.

While it was recognised that the amount of acetylcholine present in the embryo would be very small compared with choline bound as phospholipin, and since the 'Total Choline' estimated previously would include acetylcholine, it seemed desirable nevertheless to show the amount of choline linked as ester, during embryonic development.

Hestrin (1949) described a colorimetric procedure for the estimation of acetylcholine, which was merely an adaptation of the Lipmann and Tuttle (1945) technique for the assay of acyl phosphates. The method, which was previously applied to carboxylic esters by Chantrenne (1948), depends on a reaction of the ester with hydroxylamine to give a hydroxamic acid, according to the following equation:

$$R_1\text{-}CO\text{-}O\text{-}R_2 + NH_2OH = R_1\text{-}CO\text{-}NH\text{-}OH + R_2\text{-}OH$$

The hydroxamic acid gives a purple-brown colour with ferric ions.

Under the conditions prescribed by Hestrin (1949) the method gave satisfactory results over the range 150-1500 µg., acetylcholine. A solution of 300 µg. acetylcholine corresponded to an optical density/

Density of 0.20 (using the diffraction grating spectrophotometer, set to 510 mμ) and unless embryonic tissue differed vastly from most other tissues, the total amount of acetylcholine per embryo would be unlikely to exceed one-tenth of this value.

The use of more dilute reagents improved the sensitivity, but duplicate determinations of acetylcholine in pure solution, even in the range 30-180μg usually differed by 10 - 15%.

In view of these observations, it was resolved to resort to the bio-assay method of Chang and Gaddum (1933) which was found to be sensitive to 0.1 μg ester. This method involves precipitation of proteins with trichloroacetic acid (TCA) removal of acid by ether extraction, and assay of the acetylcholine in the aqueous solution by means of the contraction which it produces in the eserinised frog rectus muscle.

EXPERIMENTAL PROCEDURE.

The embryo or portions thereof, while still frozen solid was transferred to an all glass homogeniser, (Potter & Elvehjem) containing twice the tissue weight of ice-cold 10% T.C.A. The material was homogenised/

homogenised in the usual way, transferred quantitat-:ively to a chilled 100 ml. centrifuge tube using 7% T.C.A., and centrifuged at 3000 r.p.m. for 10 minutes. The supernatant liquid was decanted into a separating funnel, a similar volume of 7% T.C.A. added to the residue, and the mixture stirred . The product was centrifuged as before, the supernatant being combined with the previous one in the separating funnel. This procedure was repeated once more, but this time the residue was allowed to stand for one hour in contact with the 7% T.C.A. in the refrigerator before centrifuging. Thus from the moment of homogenising the embryo, the tissue was in contact with the extr-:actant for almost two hours, which Perry (1949) showed to be the optimum time for extraction of acetylcholine.

The supernatants were combined in the separat-:ing funnel, and extracted three or four times with an equal volume of ether, until the pH of the aqueous phase was approximately four. The solution was then concentrated <u>in vacuo</u> at 40°C to less than 2 ml., quantitatively transferred to a graduated 3 ml. tube, the pH adjusted to six, and the volume made up to 3 ml. /

In/

In the meantime a frog was pithed, and the rectus muscle mounted in a 10 ml. bath of oxygenated Ringer's solution. The muscle was stretched for two hours before use by application of a two gram weight on the lever attached to the free end of the muscle. The bath of Ringer's solution was operated at room temperature, oxygen being bubbled through the solution at about one bubble per two seconds. Before commencing a determination, the muscle was washed about six times with fresh Ringer's solution, the final volume being adjusted to the 10 ml. mark, after the addition of two drops of eserine sulphate solution (Burroughs Wellcome, 2 mg/ml) which gave a final concentration of inhibitor of approximately 1×10^{-5} Molar. Using a standard solution of acetylcholine chloride (Roche) which had a concentration of 10µg/ml a series of estimations was conducted as follows.

One minute after the addition of the inhibitor to the Ringer, the acetylcholine solution was added (say 1 µg) causing a contraction of the muscle: exactly two minutes later the muscle was stretched by a two gram weight being placed on the lever, and the solution run out of the bath. The muscle was washed six/

six times with Ringer, and after the weight had been applied for three minutes, it was removed and the inhibitor added to the bath. One minute later a new acetylcholine standard was added, and the pro-:cedure repeated as before. When several standards had been run and found to give proportional re-:sponses, an aliquot of the unknown was used in the assay. The acetylcholine standard nearest the unknown was then used, followed by the unknown, and again the same standard. When agreement to within 10% was attained between these four estimations, a 1 ml. aliquot of the unknown solution was heated at pH 12 for 15 minutes on a water-bath at 80°C., this treatment being drastic enough to hydrolyse the ester completely. The extract was then adjusted to pH 7 and its volume to 1.5 ml., and an aliquot of this solution tested for 'acetylcholine activity'. In all cases the blank obtained by this procedure (presumably due to potassium ions, free choline, etc.) was never greater than 7% of the total activ-:ity, and so no correction was applied to the results for this blank.

RECOVERY/

Recovery Experiment.

A T.C.A. extract of an unincubated egg showed no acetylcholine activity as one might expect; so to another whole egg homogenate in 10% T.C.A., 150 µg. acetylcholine was added, and the extraction procedure conducted as outlined previously. The volume of the extract was 3 ml. and the total acetylcholine activity calculated was 125 µg. This experiment showed that little acetylcholine activity was lost in the extraction procedure; in fact an 83% recovery was possible, which was considered quite satisfactory in view of the nature of the method, and properties of the assayed substance.

Correlation of acetylcholine assays with choline estimations by the reineckate procedure.

Since no absolute standard of acetylcholine was available, choline estimations by the reineckate procedure were correlated with the bio-assays of acetylcholine, prepared by the acetylation of standard choline chloride. The standard acetylcholine solution prepared by this procedure, when compared with 'Roche' acetylcholine, agreed to within ± 8% which was considered quite satisfactory.

Using/

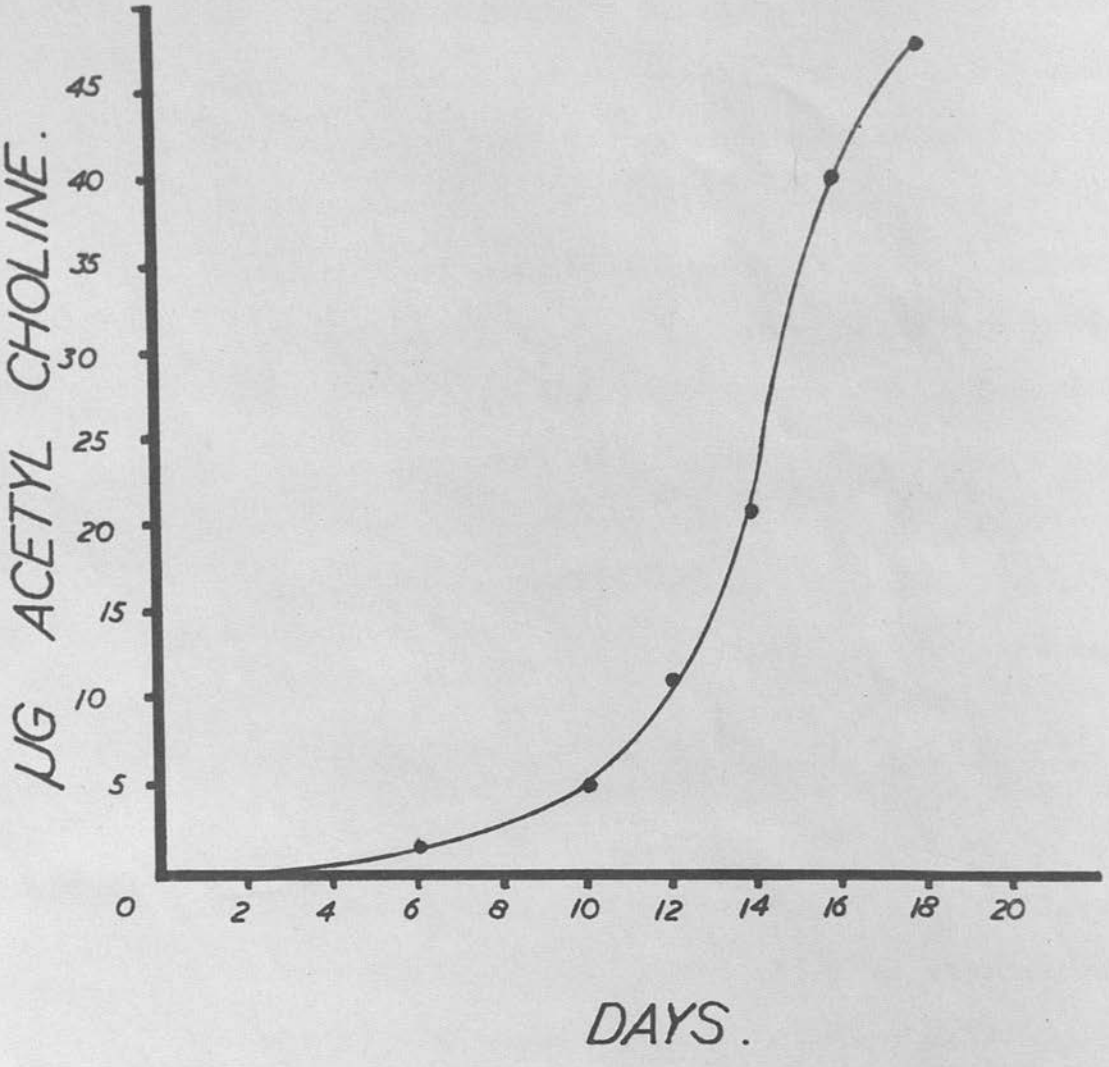

µG ACETYL CHOLINE.
45
40
35
30
25
20
15
10
5
0
2
4
6
8
10
12
14
16
18
20
DAYS.

FIGURE 9.

Using the bio-assay technique described, the acetylcholine content of embryos varying in age from six to eighteen days was estimated. In the case of the smaller embryos, it was necessary to pool a sufficient number to yield at least twenty grams (wet weight) of tissue before attempting an assay.

The results are shown in Figure 9, acetyl-:choline being plotted against incubation period.

In a number of cases a comparison was made of the results obtained in the colorimetric and the pharmacological assays. In all cases the colori-:metric procedure gave results 15-60% higher than the bio-assay, providing clear demonstration of its lack of specificity.

EXPERIMENTAL

QUALITATIVE STUDIES ON THE BASES IN THE SYSTEM

The primary object of this part of the work was to make qualitative tests for the presence of the various bases which might be intermediate pro-:ducts in the metabolism of choline. These tests for "free" bases were supplemented by experiments in which hydrolysates of the lipoid extract were similar-:ly tested. In all cases it was thought suitable to use the technique of paper partition chromatography originated by Consden, Gordon and Martin (1944) with identification of "spots" by comparison of R_F values with those of standard reference substances. It was indeed fortunate that at the very time this decision was taken, Brante (1949) should publish a modification of the original technique specially adapted to the separation of these bases. His technique was used without further modification.

Procedure

(a) "Free" bases. An extract was prepared by the method of Barger (1914). Homogenate (10 ml.) was added/

added to 200 ml. of water and heated slowly to the boiling point. The precipitate of coagulated protein was removed by filtration and washed with water. The filtrate and washings were mixed and treated with basic lead acetate. The precipitate was again discarded after filtration and the filtrate treated with H_2S to remove excess lead. The colourless filtrate from the PbS precipitation was evaporated to dryness in vacuo at 60° and the residue finally taken up in 3 ml. water. This extract contained no ions which interfered with the separation of the bases by paper partition chromatography.

Spots of this extract (about 0.02 ml.) were run on Whatman No. 4 filter paper under the conditions prescribed by Brante (1949). The solvent system placed in the trough was the "organic phase" of a mixture of butanol, acetic acid and water in the proportions 4:1:5. On the same paper, spaced about two inches apart, were run spots from solutions of the following substances: choline, ethanolamine, serine, creatinine, creatine, betaine, dimethylglycine, sarcosine, monomethylethanolamine and dimethylethanolamine. All these substances were purchased and purified except the last two. Both

these preparations and all the purifications are described in the Appendix. At the conclusion of each "run" the paper was dried and the spots developed by subliming iodine on to the paper. The deposited iodine evaporated rapidly from the paper except in the vicinity of the bases, where a brown stain persisted.

(b) The bases of the lipoid extract. Homogenate (5 ml.) was refluxed for one hour with 200 ml. chloroform-methanol (1:1). The filtered extract was divided into two equal portions, each of which was evaporated to dryness in vacuo.

One portion was hydrolysed by boiling under a reflux condenser for two hours with 30 ml. saturated aqueous $Ba(OH)_2$. Sulphuric acid (2N) was added until no further precipitation of $BaSO_4$ took place. At this stage the supernatant fluid had reached pH 3. After overnight refrigeration the precipitate was removed by filtration and the filtrate concentrated to 3 ml. in vacuo.

The second portion of lipoid extract was hydrolysed for two hours under reflux with 20 ml. approximately 4N methanolic HCl. The hydrolysate was/

was chilled overnight in the refrigerator, filtered, and the filtrate evaporated to dryness in vacuo at 60°. Distilled water (25 ml.) was added to the residue and the evaporation in vacuo repeated. Finally, the residue was taken up in 3 ml. water.

Spots (about 0.02 ml.) of both the acid and alkaline hydrolysates were run on Whatman No. 4 paper exactly as for the "free" bases, and the same standard substances were run for comparison.

Results

These techniques were applied to homogenates from both embryos and "remainders" at 12, 16 and 20 days incubation. The stage of incubation made no difference to the results. The lipoid extracts of the "remainders" gave only two apots, corresponding to choline and ethanolamine, while those of the embryos gave these two and an additional one which was identified as serine. It would thus appear that egg yolk contains only the phospholipins phosphatidyl choline and phosphatidyl ethanolamine, and that to these is added in the embryo the phosphatidyl serine discovered by Folch (1941)

The/

The extracts containing the 'free' bases in the 'remainder' showed the presence of creatine and creatinine, because the 'remainder' included the allantoic fluid, into which the embryo excreted these substances. The embryos showed clear indications of much creatine and traces of creatinine and choline and betaine. No further spots were found, except a slow running, rather diffuse spot almost at the initial datum line, which was probably due to chol-:esterol and sphingosine; this finding seemed to support the view that any other bases which were present, must be there in concentrations too low to be detected by paper partition chromatography. The R_f values of betaine and dimethylaminoacetic acid were found to be very similar in all the solvent mixtures used, and so it was necessary to make special attempts to separate these compounds. Allowing the solvent front to run off the paper for 48 hours caused the separation of these two substances in pure solution. The experimental 'betaine' spot, however, only became irregular and elongated.

The/

The possibility has therefore not been satisfactorily excluded that the traces of betaine may be contaminated by an even smaller trace of dimethylaminoacetic acid.

The presence of traces of betaine lends colour to the possibility that this substance may be an intermediate compound in the metabolism of choline, and raises interest in the enzyme choline oxidase, which was the subject of experiments reported in the next section.

EXPERIMENTAL

Enzyme Studies:

It is almost certain that the great majority of the reactions involved in the metabolism of choline (and methyl groups in general) must be catalysed by enzymes. Experiments already described suggest that two of these reactions may be oxidation of choline to betaine and transmethylation from betaine as a methyl donor. This section describes experi-:ments on the enzymic catalysis of these reactions, and a few limited experiments on one carbon fragments.

Choline Oxidase:

The enzyme choline oxidase, originally discov-:ered by Bernheim and Bernheim (1933), catalyses the oxidation of choline to betaine. As a result of further work carried out by the discoverers in 1938, and by Mann, Woodward and Quastel (1938), it is known that the enzyme is present in the liver of many animals but absent from brain, blood, spleen, heart and skeletal muscle; that the reactions take place in two stages, with betaine aldehyde as an intermediate product; that the enzyme is not usually obtainable in aqueous extracts, but remains in the insoluble residue; that the enzyme depends for its activity on the presence of intact sulphydryl groups/

groups; and that the enzyme is inhibited by quaternary nitrogen ions and by long-chain fatty acids. The last fact may be of some physiological importance in that it provides a possible mechanism for the alleviation of a fatty liver: inhibition of choline oxidase might result in the availability of more choline for phospholipin synthesis and fat transport. The suggestion has also been made (Dinning, Keith and Day, 1950) that in monkey livers folic acid may be a co-enzyme for choline oxidase. As all factors regulating the activity of the enzyme appeared to have some relevance to the present work, this idea was tested experimentally on the chick liver preparations. The possibility that Co-enzyme I might function as an electron-acceptor for this system was also tested.

Methods:

Livers were excised from chick embryos varying in age from 14 to 20 days, and in a few experiments from six day old chicks. Generally the livers from a group of embryos of the same age were pooled, and at once homogenised with twice the liver/

liver weight of M/15 phosphate buffer, pH 7.8, in a homogeniser (Potter and Elvehjem, 1936) cooled in ice water. The homogenate was strained through muslin, the filtrate diluted with an equal volume of ice cold water, and centrifuged at 2500 r.p.m. for 10 minutes. The supernatant was discarded and the residue stirred up with M/30 phosphate buffer, pH 7.8, and centrifuged as before. The resulting pale brown residue was mixed with an equal volume of M/15 phosphate buffer, and stored in the refrigerator until required.

Experiments to demonstrate choline oxidase activity in this suspension were conducted in the conventional Warburg apparatus at pH 7.8. The "well" of the flask contained 0.2 ml. 10% potassium hydroxide to absorb carbon dioxide, while the annulus contained the other reagents, namely, an aliquot of the enzyme suspension (0.5 ml.), 3 mg. choline chloride substrate (0.5 ml.) and phosphate buffer, pH 7.8 (1.2 ml.). Other factors (such as folic acid, etc.) which were postulated as influencing the enzymic activity, were added together with the buffer, to maintain the total fluid volume in the flasks at 2.4 ml. Oxygen uptake measurements were/

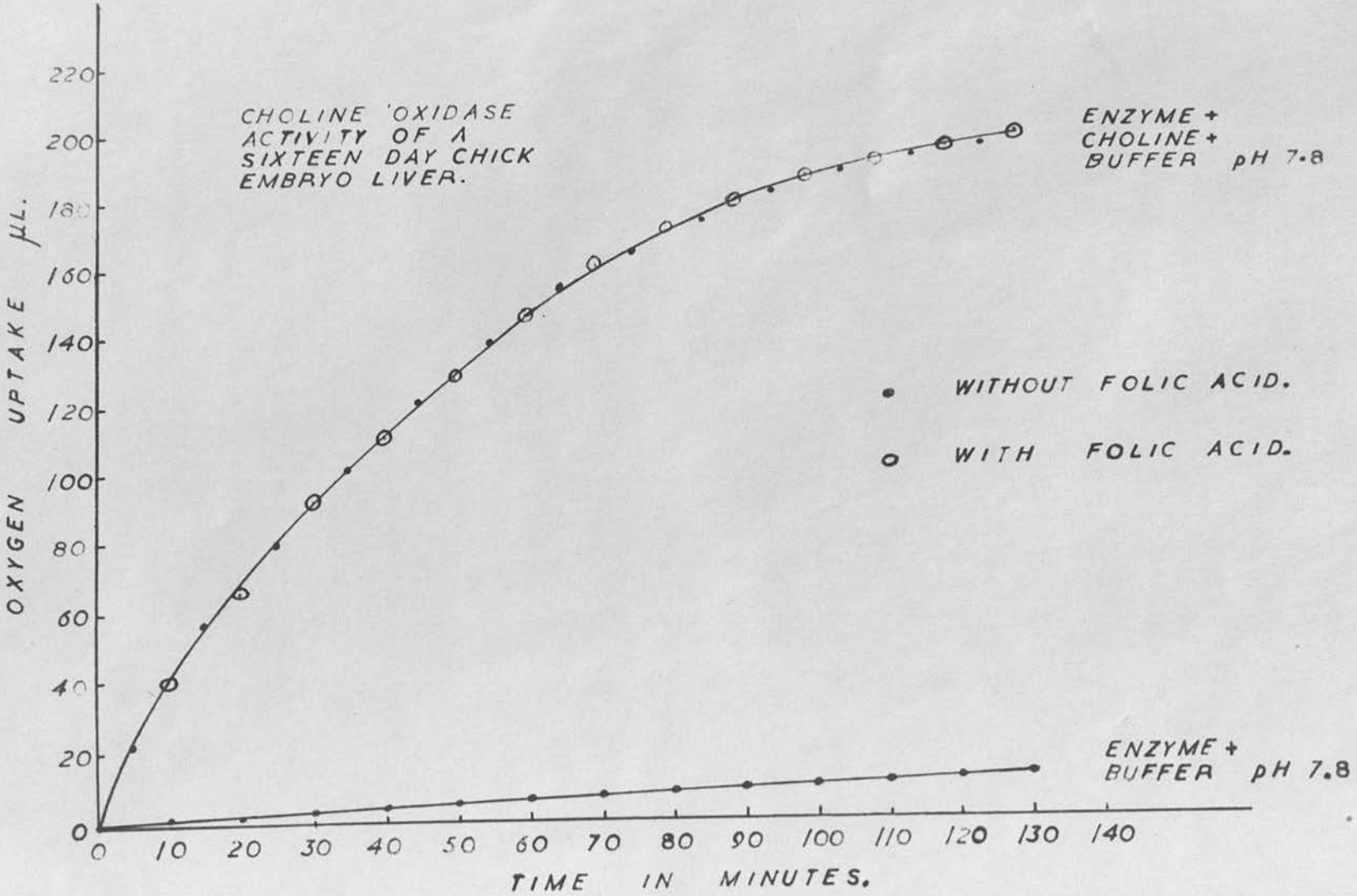

CHOLINE 'OXIDASE ACTIVITY OF A SIXTEEN DAY CHICK EMBRYO LIVER.
ENZYME + CHOLINE + BUFFER pH 7.8
WITHOUT FOLIC ACID.
WITH FOLIC ACID.
ENZYME + BUFFER pH 7.8
OXYGEN UPTAKE µL.
TIME IN MINUTES.
0
20
40
60
80
100
120
140
160
180
200
220
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140

FIGURE 10

were taken every 5 minutes for about two hours, the gaseous phase being pure oxygen, and the temperature of the water bath 37°C.

In all the experiments, both the "enzyme blank" (enzyme without substrate) and the "substrate blank" (boiled enzyme and substrate) consumed little oxygen. Typical oxygen uptake graphs are shown in Figures 10 and 11, which show that the "blanks" used approximately 10% of the oxygen consumed in the reaction vessels.

Action of Folic Acid on Choline Oxidase 'in vitro'.

In order to test the hypothesis of Dinning et al.(1950) that folic acid acts as a co-enzyme in the choline oxidase system, this substance (L.Light) was added to the reaction mixtures in amounts varying from 100-1000 µg. and showed no effect (see Figure 10). Thus, folic acid in these quantities had no co-enzyme-like action. This finding has recently been confirmed by Williams et al. (1950).

Action of D.P.N. on Choline Oxidase 'in vitro'.

During further studies on this enzyme it was decided to test whether diphosphopyridine nucleo-

tide/

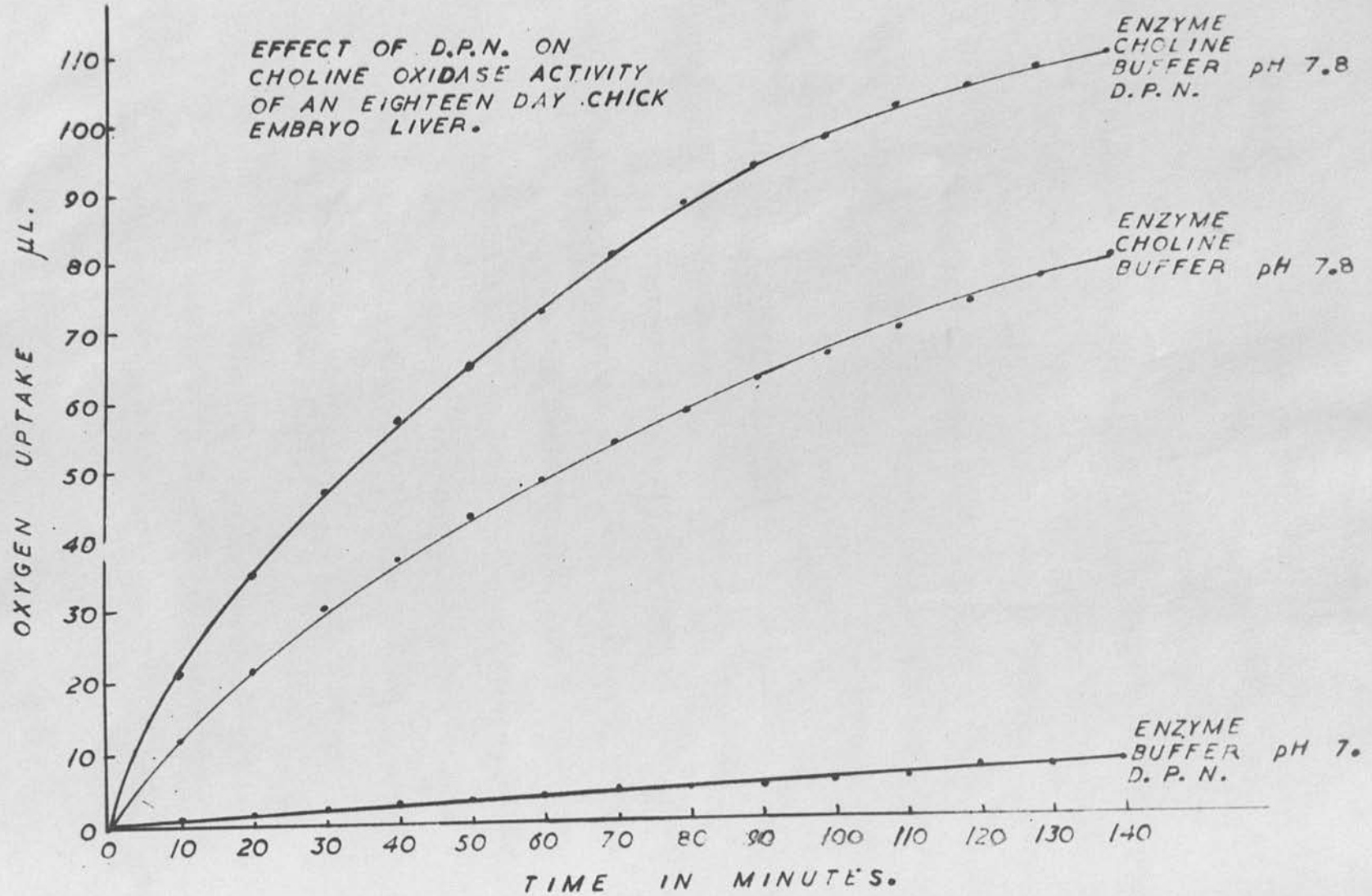

EFFECT OF D.P.N. ON
CHOLINE OXIDASE ACTIVITY
OF AN EIGHTEEN DAY CHICK
EMBRYO LIVER.
ENZYME
CHOLINE
BUFFER pH 7.8
D.P.N.
ENZYME
CHOLINE
BUFFER pH 7.8
ENZYME
BUFFER pH 7.
D.P.N.
OXYGEN UPTAKE µL.
0
10
20
30
40
50
60
70
80
90
100
110
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
TIME IN MINUTES.

FIGURE 11

:tide (D.P.N.) affected the rate of the reaction. D.P.N. kindly prepared by Dr. J.K. Grant according to the method of LePage (1949), was dissolved in phosphate buffer, to yield a solution containing 1 mg./ml. The addition of 300 µg. of this material caused an increase in the rate of oxygen uptake, showing that the enzyme was capable of utilising D.P.N. as an electron acceptor (see Figure 11). It is interesting to note that the dialysed enzyme preparation was entirely inactive and D.P.N. could not reactivate the system showing that some other dialysable factor was also involved in this system.

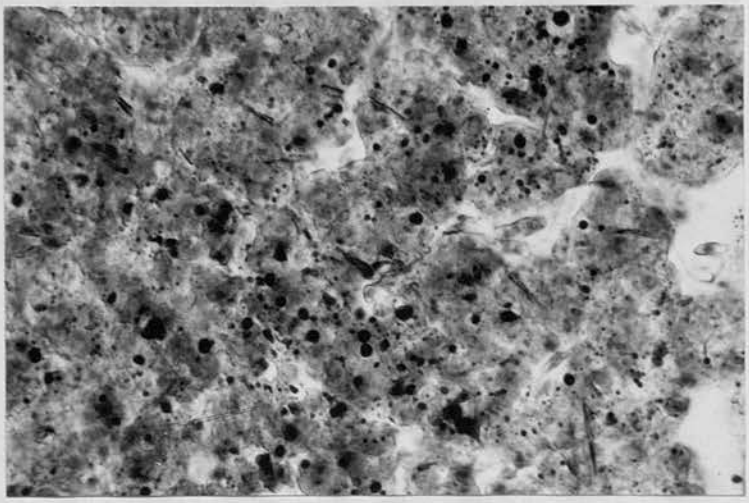
Characterisation of the reaction product.

To demonstrate that the oxygen uptake observed in these experiments was due to the oxidation of choline to betaine, the following experiment was performed. About 25 mg. choline chloride in 30 ml. phosphate buffer (pH 7.8) was incubated with 2 ml. enzyme preparation in a 250 ml. conical flask for 2 hours at 37°C under oxygen. The protein was then precipitated with trichloroacetic acid, centrifuged, and the supernatant extracted several times with ether to remove this precipitant. The aqueous phase was finally concentrated to less than 1.0 ml./

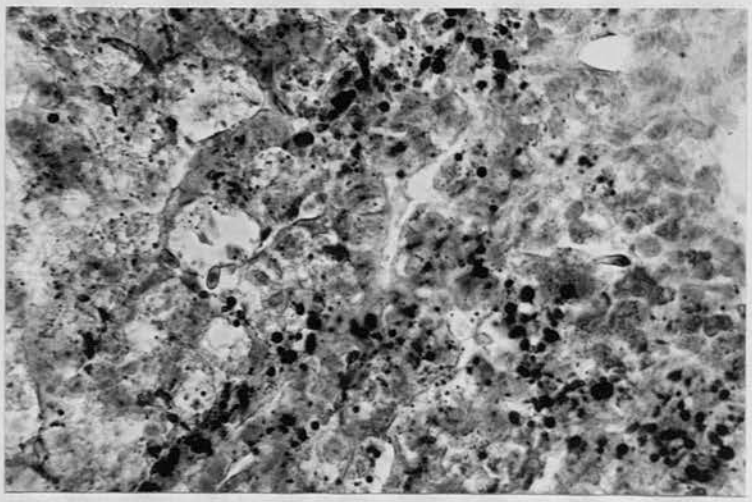
1.0 ml. and one drop of this solution was run on a paper partition chromatogram with the same solvents and technique as previously described (Brante, 1949). Reference spots of choline and betaine were also placed on the same paper, and the solvent front was run off the paper for two days. The dried paper showed under the usual iodine development, that the only bases present in the unknown solution were choline and betaine. This supported the hypothesis that the oxygen uptake observed was due to oxidation of choline to betaine.

These experiments showed that the embryo chick liver had choline oxidase activity, but although quantitative estimations of the enzymic activity were not required for the purpose of this work, one "semi-quantitative" observation did emerge. The embryo liver had a normal appearance up to the sixteenth or seventeenth day, after which the fat content steadily increased up to the time of hatching, when it appeared enlarged and yellow. These fatty livers observed in late embryos and in day old chicks had low choline oxidase activity; this is in accordance with the observation that long-chain fatty acids inhibit this enzyme. Figures 12, 13, and 14 are photomicrographs from/

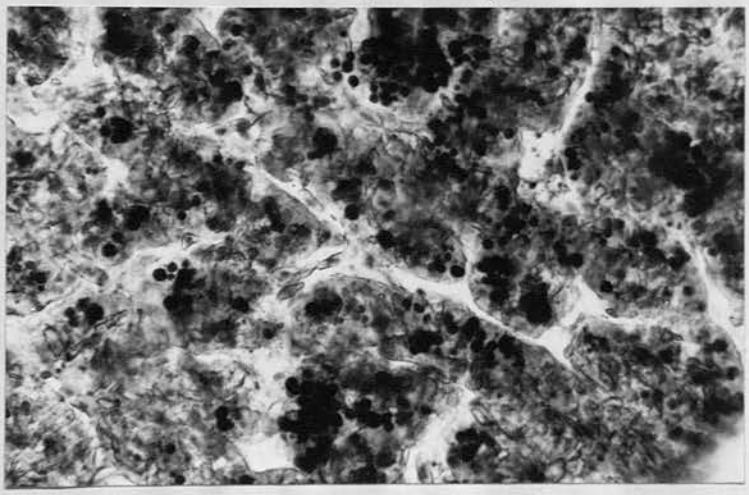
PHOTOMICROGRAPHS OF EMBRYO CHICK LIVER



13 day embryo



17 day embryo



21 day embryo

Stain: Scharlach R

x 420.

from livers of 13, 17 and 21 day chick embryos respectively, and indicate clearly the gradual appearance of the characteristic fatty infiltration during embryonic development.

The development of the fatty liver seems remarkable in view of the large quantities of choline and methionine available to the embryo. There would appear to be some support here for the ideas of Chaikoff et al. (1945) and Dragstedt et al. (1939), who believe that certain factors other than those hitherto investigated are involved in the fatty liver problem. This onset of a fatty liver under physiological conditions deserves further investiga-:tion.

Transmethylase:

A series of papers by Borsook and Dubnoff (1947, 1948) suggests the existence of a number of transmethylases, each specific with respect to the methyl donor; whether the specificity also extends to the methyl acceptor is not known.

The analytical results described earlier (page 39) prove that creatine is synthesised in the chick/

chick embryo, but the physical and chemical proper-:ties of glycocyamine have made it impossible to demonstrate the synthesis of creatine 'in vitro' from glycocyamine and betaine in the presence of a liver preparation. It has however been possible to demonstrate the enzymic synthesis of methionine from homocysteine and betaine by following closely the technique of Dubnoff (1949) as follows.

Method:

A number of livers from embryos of the same age were 'pooled' and homogenised with an equal volume of phosphate buffer at pH 7.4, and incubated with betaine and homocysteine for two hours at 37°C under oxygen-5% carbon dioxide. The mixture was usually 1 ml. homogenate, 4 ml. phosphate buffer, pH 7.4, 10 mg. homocysteine and 10 mg. betaine. Betaine was used in place of choline because it had already been shown that this almost identical liver preparation could oxidise choline to betaine. After two hours incubation the methionine content of a trichloroacetic acid filtrate was estimated by Dubnoff's (1949) modification of the McCarthy and Sullivan (1941) procedure. Thus it was possible to demonstrate the 'in vitro' synthesis of methionine as shown in Table 1.

Table/

Table 1.

Enzymic synthesis of Methionine from Homocysteine and Betaine.

No. Mixture	Reading at 520 mμ	diff. from No.1.	Methionine synthesised. (mg.)
1. Liver homogenate	0.052	-	-
2. Liver homogenate and Betaine	0.068	0.016	0.11
3. Liver homogenate Betaine and Homocysteine	0.104	0.052	0.36
4. Liver homogenate and Homocysteine	0.071	0.019	0.13
5. Boiled Liver homogenate and Betaine and Homocysteine	0.061	0.009	0.06

These experiments demonstrate that the liver of the embryo chick provides at least two of the enzymes catalysing reactions which are generally believed to be of importance in 'Methyl Group' metabolism in other animal systems.

Additional Enzyme Studies.

Within recent years certain profound changes have occurred in our concepts of the substances involved in intermediary metabolism. Handler et.al. (1941) were first to report the occurrence of formaldehyde in mammalian tissue. These workers found that a rat liver preparation was capable of oxidatively demethylating sarcosine (and dimethylaminoacetic acid) to formaldehyde and glycine, detecting formaldehyde by a colour reaction. Recently MacKenzie (1950) has confirmed this reaction by using sarcosine labelled with ^{14}C in the methyl group, which he incubated with a rat liver homogenate, and then isolated ^{14}C labelled formaldehyde as the dimedon derivative. In the light of these results, it seemed possible that the suspected by-product of a transmethylation from betaine, namely dimethylamino-acetic acid, might be metabolised along this oxidative demethylation pathway.

Attempts were made to detect formaldehyde, either in an embryonic homogenate, or in a homogenate of embryonic liver tissue, using a steam distillation technique. The homogenates were steam-distilled in

a/

a micro-Kjeldahl apparatus into sodium sulphite, and an aliquot of this solution was treated with chromo-:trophic acid in 9M sulphuric acid, according to the method of MacFadyen (1945). Neither the embryo homogenate nor the embryo liver homogenate yielded any significant quantity of formaldehyde.

Further experiments were attempted incubating at 37°C sarcosine or dimethylaminoacetic acid with embryo liver homogenate at pH 7.0 for one hour, followed by steam distillation of the volatile products as before. Once again it was found to be impossible to detect formaldehyde, and so it must be assumed that if formaldehyde was produced, its rate of oxidation to formic acid must exceed the rate of production. Experimental proof of this rapid oxidation of formal-:dehyde to formate by rat liver has been obtained by Bernheim (1950). It therefore seemed impossible to demonstrate the oxidative demethylation of dimethylaminoacetic acid by embryo chick liver tissue unless one had isotopic carbon available.

Utilisation/

Utilisation of Formate

Using ^{14}C labelled formate and glycine, Siekevitz and Greenberg (1949) have shown that rat liver preparations could synthesise serine with the β carbon atom labelled with ^{14}C. Attempts were made to demonstrate the synthesis of serine by chick liver homogenates from glycine and formate, using the procedure of Siekevitz and Greenberg (1949). The only difference between the two methods was that whereas Siekevitz et al. added an excess of non-isotopic serine as a carrier for isolation purposes, the technique employed in this laboratory was attempted identification of serine by paper partition chromatography, followed by development with ninhydrin. After many attempts it was found to be impossible to identify with certainty serine in a protein-free filtrate by this technique. It was felt that as serine could not be detected by this method there was no point in attempting any of the usual methods for serine estimation, and therefore the experiments were abandoned.

Choline/

Choline oxidase activity has been demonstrated in embryo chick liver and at least two transmethyla-:tions are known to take place. Using non-isotopic methods it was found to be impossible to demonstrate in the embryo chick liver, either the oxidative demethylation of dimethylaminoacetic acid to formaldehyde and glycine, or the condensation of formate and glycine to yield serine. It is not intended to imply that these reactions which have been demonstrated by isotopic techniques to occur in rat liver do not occur in embryo chick liver, but only to record the author's failure to observe these reactions by non-isotopic methods.

DISCUSSION

As a consequence of the experimental work detailed in this thesis, involving estimations of certain quantitatively important methylated compounds in the developing chick embryo and in the 'remainder', together with certain enzyme studies, the following conclusions appear valid.

In the system as a whole, the methionine content remains constant until about the seventeenth day of incubation whereupon the amount of this sub-:stance decreases until at the moment of hatching there remains approximately 90% of the original methionine. Therefore, methionine is not acting as a source of 'labile' methyl groups until about the seventeenth day of incubation. Whether before the seventeenth day methionine was involved in transmeth-:ylation mechanisms in a catalytic role (see page 14) is not known, but it can be stated that up to the seventeenth day methionine is not the ultimate methyl donor.

The choline content of the system on the other hand decreases fairly steadily until at the end of incubation there is only 50% of the initial amount of choline. This shows that the embryo chick/

chick actively metabolises choline, and one problem is to ascertain the fate of the choline metabolised.

During embryonic development creatine, in common with many other constituents (choline, methionine, etc.), accumulates in the embryo body in a roughly exponential manner with respect to the incubation period. There would appear to be little significance in this exponential accumulation of various substances in the embryo body, because the 'wet weight' of the embryo increases logarithmically, and this 'wet weight' could be taken as a rough approximation of the cell number. Some creatine is excreted by the embryo into the allantoic fluid, so that the creatine content of the 'remainder' also increases. This system as a whole contains very little creatinine.

Presumably, therefore, until the seventeenth day of incubation (while the methionine content is still constant) creatine is being synthesis ed by the methylation of glycocyamine at the ultimate expense of choline methyl groups.

Isotopic studies by Muntz (1950) have con-:firmed the theory put forward by Dubnoff (1949) that choline must be oxidised to betaine before losing its one/

one 'labile' methyl group. Since choline seems to be the principal methyl donor in the system under consideration, it was necessary to establish whether a mechanism was available for the conversion of choline to betaine.

The embryonic chick liver was found capable of oxidising choline to betaine and therefore appears to contain the enzyme choline oxidase. If this oxidation step is essential in order to 'labilise' the methyl group in choline, then all factors which affect the activity of choline oxidase will control transmethylations from choline.

Dinning et al (1950) suggested that folic acid may be a co-enzyme in this choline oxidase system, but 'in vitro' studies using embryo chick liver choline oxidase have failed to confirm this view (cf. Williams et al, 1950). It seemed rather surprising that Dinning et al from their 'in vivo' studies on inhibition of choline oxidase by folic acid antagonists should put forward the theory that folic acid might be a co-enzyme for choline. Embryo chick liver choline oxidase 'in vitro' has been found to be capable of utilising diphosphopyridine nucleotide as an electron acceptor.

Borsook/

Borsook and Dubnoff (1948) have demonstrated the existence of a transmethylase enzyme required in biological methylation of glycocyamine and homocys-:teine, using betaine as the methyl donor. An embryo chick liver homogenate on incubation with betaine and homocysteine was found to synthesise methionine, showing that the preparation contained at least one transmethylase enzyme. Presumably since large quantities of creatine are synthesised by the embryo 'in vivo', the transmethylase involved in the methylation of glycocyamine must also be present. Thus, the developing chick embryo had both choline oxidase and possibly two transmethylases, showing that the system was capable of utilising choline as an ultimate methyl donor.

Using the values obtained in the analyses , assuming that both choline and methionine have one labile methyl group per molecule, and including the methyl group in creatine, it was possible to effect a summation of 'labile' methyl groups at various stages of embryonic development. This 'balance sheet' of 'labile' methyl groups shows that during incubation at least 25% of the 'labile' methyl groups are "lost" and hence are metabolised along some as yet unknown pathway.

Since/

Since most of this methyl group "loss" is due to a decrease in total choline in the system, it seems reasonable to seek an explanation of the methyl group disappearance in terms of 'choline decrease'. All the choline estimations were conducted by the reineckate precipitation.procedure, and while this method is not absolutely specific for the quaternary base - choline, most reineckates of other quaternary bases (such as betaine) are much more soluble, and hence these substances would not interfere with the estimation of choline. This means that if choline were merely oxidised to betaine (by choline oxidase) then this process would be recorded as a choline decrease and then appear on the balance sheet as a methyl group loss, even although betaine was accum-:ulating in the system.

Studies on the bases occurring in the system revealed that betaine was only present in minute traces, and it did not seem conceivable that the 'labile' methyl group 'loss' was due to this oxida-tion procedure, and hence the explanation must be sought elsewhere.

If one accepts the view that choline must be oxidised to betaine before losing a methyl group (Dubnoff, 1949; Muntz, 1950) and also the hypothesis that only one methyl group per molecule of choline or betaine/

betaine is capable of participation in a transmethyl-:ation reaction ('labile'), then the obvious conclus-:ion is that the by-product of this transmethylation is dimethylaminoacetic acid. This substance was not detected in the base studies previously mentioned, and since the methyl groups in this compound are considered to be 'non-labile' it must be metabolised by some other mechanism.

Handler et al (1941) showed that rat liver preparations could oxidatively demethylate this sub-stance, dimethylaminoacetic acid, to formaldehyde and glycine. Using a similar embryonic chick l iver preparation it was not possible to demonstrate oxidative demethylation of this compound as measured by formaldehyde production. This result might mean that if embryonic chick liver tissue does metabolise dimethylaminoacetic acid, then formaldehyde is not an intermediate; or that the metabolic pathway does in-:volve formaldehyde, but this compound is rapidly oxidised to formate.

As most of the 'labile' methyl groups which are lost to the system have their origin in choline, it is interesting to speculate whether the 'lost' choline is initially metabolised through the choline oxidase enzyme system to yield betaine. This com-:pound, only traces of which were detected in this system/

system, might lose a methyl group to some, as yet unknown, acceptor, and the by-product of the reaction would once more be dimethylaminoacetic acid.

This theory presupposes that all the choline which is metabolised in this system utilises the enzyme choline oxidase for conversion to betaine, and after acting as a methyl (or precursor of a "One carbon fragment") donor the remaining substance (dimethylaminoacetic acid) might possibly be oxida-:tively demethylated to two molecules of formaldehyde (or two "One carbon fragments") and glycine.

Siekevitz and Greenberg (1949) showed that ^{14}C formate and glycine 'in vitro' could yield serine labelled in the β-carbon atom. This pathway to the β-carbon atom of serine is only one of the many now well established reactions in which formate, formal-:dehyde, or the "One carbon fragment" participates. It seems conceivable that the object of metabolising the "lost" choline was to produce "One carbon frag-:ments" required in certain bio-syntheses.

The histological evidence regarding the onset of a marked fatty infiltration of the liver appears to coincide with the decline in the total methionine. Choline oxidase is known to be inhibited in fatty livers (Handler et al, 1942) and if choline must be oxidised/

oxidised before acting as a methyl donor, then inhibition of this enzyme would result in inactivation of choline as a methyl donor. This might account for the embryo after the onset of the fatty liver condition at about the seventeenth day of incubation, drawing on its methionine reserves for use in biological methylation.

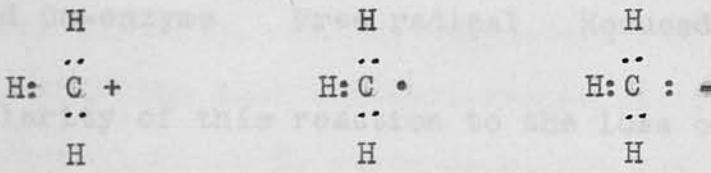
The onset of this fatty liver condition seems most surprising in view of the relatively large quantities of choline and methionine (in the "remainder") still available to the embryo even at the point of hatching. This would appear to support the hypothesis of Chaikoff et al. (1945) and Dragstedt et al. (1939) who are in agreement that lipotropic factors other than choline, betaine and methionine are involved in the fatty liver problem. Further studies on the nature of the lipotropic factors involved in the relief of this condition, which normally occurs in the first week of the chick's life, would probably be worthy of investigation.

The Mechanism of Transmethylation.

No discussion of transmethylation would be complete without some reference to the mechanism of this remarkable biochemical reaction, in which only certain/

certain N-methyl and S-methyl compounds participate. Although the experimental work described here contributes nothing directly to this aspect of the matter, there are certain theoretical considerations which it seems wise to present because they have been overlooked in the past, and they might be the basis of further study on this subject.

N-methyl Compounds. When a methyl group is transferred from (say) betaine to some acceptor molecule, it may migrate as a positive ion (Challenger, 1945), as a free radical, or as a negative ion, thus:-



Positive ion Free radical Negative ion

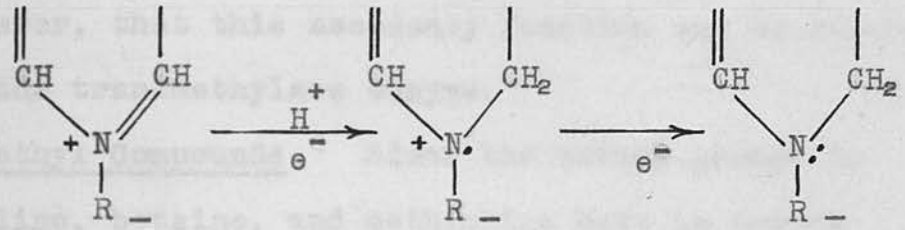
The positive ion hypothesis was advanced by Challenger in 1945, based on the interpretation by Hughes et al. (1933) of pyrolytic demethylations.

The second possibility, that the free radical is the active intermediate does not appear to have been considered by other workers.

Free radicals have been shown to play an important part in biological oxidation processes, and a close parallel can be drawn between the reduction/

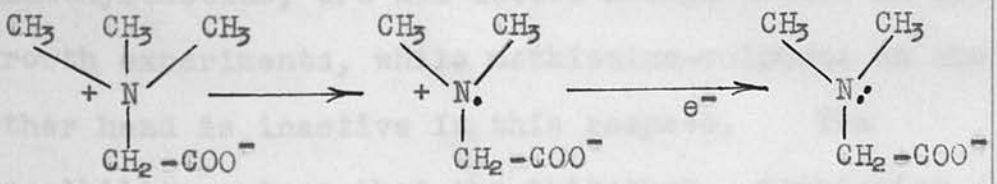
reduction of Co-enzymes I and II and the fate of betaine in a transmethylation reaction.

In both cases there is a change from a quadri-:covalent unipositive nitrogen ion, to a tercovalent nitrogen atom. In the case of the phosphopyridine nucleotides it is known that there is an intermediate free radical stage, the reaction usually being represented as follows:



Oxidised Co-enzyme Free radical Reduced Co-enzyme

The similarity of this reaction to the loss of a methyl group by betaine is so striking that it is tempting to suggest that this latter reaction also proceeds through a free radical stage, thus:



Free radical

It will be apparent that the splitting of betaine to yield this free radical would of necessity also cause the formation, at least transiently, of a free methyl radical/

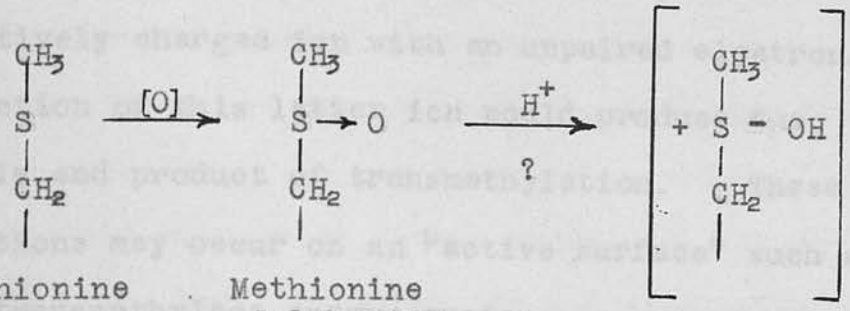
radical, which might be available for reaction with a suitable acceptor molecule.

A criticism of this hypothesis could be based on the concept that the free radical produced during the reduction of the phosphopyridine nucleotides is stabilised by the presence of the pyridinium nucleus, while no such stabilising influence is apparent in the betaine molecule. The possibility remains however, that this necessary function may be discharged by the transmethylase enzyme.

S-methyl Compounds. Since the methyl groups in choline, betaine, and methionine have in common biological lability, and the suggestion has been advanced that the labile methyl groups participate in transmethylations by means of a free radical mechanism, it remains to extend this theory to the S-methyl compounds. Methionine, Methionine-methyl -sulphonium salts, methionine sulphoxide, and dimethylthetins, are all active methyl donors in rat growth experiments, while methionine-sulphone on the other hand is inactive in this respect. The possibility arises that the thioether - methionine - must be converted into either methionine sulphoxide, or a methionine-methyl-sulphonium salt, before acting as a methyl donor.

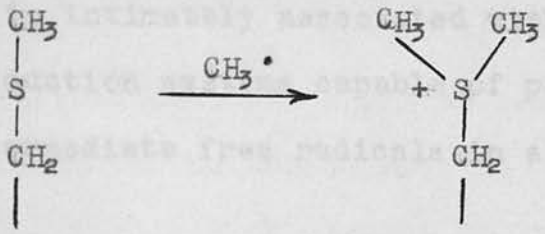
This/

This would mean conversion of a bivalent sulphur atom into a tervalent sulphur ion by oxidation, as follows:



Methionine Methionine Sulphoxide.

or, by methylation thus:



Methionine Methionine-Methyl sulphonium salt.

Either preliminary step would alter the molecule of methionine into a positive ion, analogous to the quaternary base betaine. Thus from this point, the mechanism of loss of a methyl group as a free radical from the methionine derivative might be a closely similar process to that envisaged for the loss of a methyl group from quaternary nitrogen compounds, thus:

Loss/

Loss of a free methyl radical from a positively charged methyl sulphonium compound to yield a positively charged ion with an unpaired electron. Reduction of this latter ion would produce the stable end product of transmethylation. These reactions may occur on an "active surface" such as the transmethylase enzyme system.

As a consequence of this hypothesis, it is necessary to assume that the mechanism of transmethylation is intimately associated with suitable oxidation-reduction systems capable of participating with the intermediate free radicals in a reversible reaction.

SUMMARY

1. Determinations were made of certain quantitatively important methylated compounds in the embryonated hen egg, namely, choline, methionine and creatine. It was shown by experiment that it would be safe to ignore creatinine, acetylcholine, betaine, sarcosine and dimethylaminoacetic acid. The absence of other N-methylated compounds in quantitatively important concentrations was established.

2. Choline, methionine, and creatine were all found to accumulate in the embryo body in broadly exponential manner with respect to incubation period.

3. In the system as a whole the methionine content remains constant until about the seventeenth day of incubation, after which it decreases to 90% of the original amount. Whilst methionine may be involved in transmethylations in a catalytic capacity (see page 14) it cannot be acting as the ultimate source of labile methyl groups until after the seventeenth day of incubation.

4. In the system as a whole, choline decreases steadily throughout incubation to about half the initial/

initial amount. Of the methyl groups associated with the choline which disappears, not more than 30% can be accounted for by observed creatine synthesis. The choline "lost" to the system must be metabolised along some route not involv-:ing the recognised well known transmethylation reactions.

5. Summation of "biologically labile" methyl groups at various stages throughout incubation revealed that 25% of the "labile" methyl groups originally present in the system "disappeared" during incubation.

6. Embryonic chick liver tissue 'in vitro' has been found to contain an enzymic mechanism capable of oxidising choline to betaine. This system is unaffected by folic acid (cf. Williams et al, 1950), but can utilise diphosphopyridine nucleotide as an electron acceptor.

7. The marked appearance of a fatty liver after the seventeenth day of incubation was accompanied by a decrease in choline oxidase activity, presumably due to inhibition of this enzyme by neutral fat and fatty acids.

8. Embryonic/

8. Embryonic chick liver tissue 'in vitro' was found to be capable of effecting the synthesis of methionine from homocysteine and betaine; hence, there appears to be a transmethylase present. Also, since an overall creatine synthesis was demonstrated, there may be more than one trans-:methylase, depending on the degree of specificity of such enzyme systems.

9. The by-product of a transmethylation from betaine should be dimethylaminoacetic acid. While this substance may be present in traces, it did not accumulate in this system. Efforts to demonstrate the existence of a catabolic pathway of metabolism to formaldehyde and glycine (Handler et al, 1941) have been unsuccessful.

10. Recent isotope studies have shown that 'labile' methyl groups may be used for the synthesis of a number of non-methylated compounds, eg. serine. In an endeavour to explain the 'labile' methyl group loss, it was thought that they might be metabolised first to formaldehyde, formate or a "One carbon fragment". Attempts were made to demonstrate/

demonstrate the synthesis of serine from glycine and formate, but these experiments were again unsuccessful.

11. The most important conclusion to be drawn from this work is that at least in the chick embryo, recognised transmethylation reactions do not account for more than one-third of the choline metabolised. This is in accord with recent isotope studies in the rat, which show that the methyl group occupies a much more important position in the general metabolic field than was formerly believed.

APPENDIX I

This appendix describes the more important experiments made in order to check the efficiency and reliability of the extraction procedure, and analytical methods employed.

Lipoid Extraction

Two uncertain matters were tested in connection with the lipoid extraction:

(a) The number of extractions required to remove phospholipins completely from the protein precipitate.

(b) The relative efficiency of chloroform-methanol (1:1) and ethanol-ether (3:1) as extracting solvents.

In both cases it was felt that the total amount of phosphorus extracted by the lipoid solvent would provide a suitable index of phospholipin content. The phosphorus determinations were made by a delicate and highly reproducible modification of the molybdenum blue method (Allen, 1940). The optical measurements were made on a D.G. Spectrophotometer set to a wavelength of 700 mμ .

(a) The/

(a) The number of extractions required (using chloroform-methanol.

A number of extractions of a single sample of homogenate (2 ml.) were made in the usual way (see page 29), and the total amount of phosphorus in each separate extract determined. The results are recorded in Table i.

Table i.

The number of Extractions required to remove all the lipoid material

Extraction Number	Vol. of Extract ml.	P. per Extract µg.	P. as % of Total	Sum Total Extracted %
1	20	2400	82.0	82.0
2	"	420	14.5	96.5
3	"	49	3.3	99.8
4	"	9	0.16	99.96
5	"	2	0.04	100.
6	"	0	-	-
7	"	2	-	-

This experiment showed that four extractions with 20 ml. chloroform-methanol extracted all the phospholipin.

(b)/

(b) To ascertain the most suitable lipoid solvent.

To assess the relative efficiency of the two solvent mixtures, Chloroform-methanol (1:1) and ethanol-ether (3:1), aliquots of a homogenate (2 ml.) were separately extracted with four 20 ml. portions of each solvent (see page 29). The extracts obtained with each solvent were combined and made up to 100 ml. Duplicate 2 ml. samples of each solution were analysed for phosphorus, the results being recorded in Table ii.

Table ii.

Comparison of Chloroform-methanol and Ethanol-ether as Lipoid Extractants

Solvent	Vol. of Homogenate ml.	Extractions	Total P. Extracted µg.	Mean µg.
Ethanol-ether 3:1	2	4 x 20	2775	
	2	4 x 20	2765	2770
Chloroform-methanol 1:1	2	4 x 20	2825	
	2	4 x 20	2815	2820

It was decided to continue using chloroform-methanol as the lipoid solvent, as this experiment indicated that the two solvent mixtures gave closely similar results.

EXPERIMENTS TO CHECK THE RELIABILITY OF THE CHOLINE METHOD

1. Quantitative liberation of choline from the phospholipin fraction.

Before choline can be determined in a solution of phospholipins, these compounds must be hydrolysed. Barium hydroxide was used in this work because it is a relatively mild reagent and it is easy to remove excess barium ions (as sulphate) after the hydrolysis. The following experiment was conducted in order to establish the rate of liberation of choline from the mixed phospholipins of an unincubated egg.

Aliquots (10 ml.) of a lipoid extract were taken to dryness in 15 ml. graduated tubes and the residue treated with 3 ml. saturated aqueous barium hydroxide at 100°C. At fixed intervals after immersing/

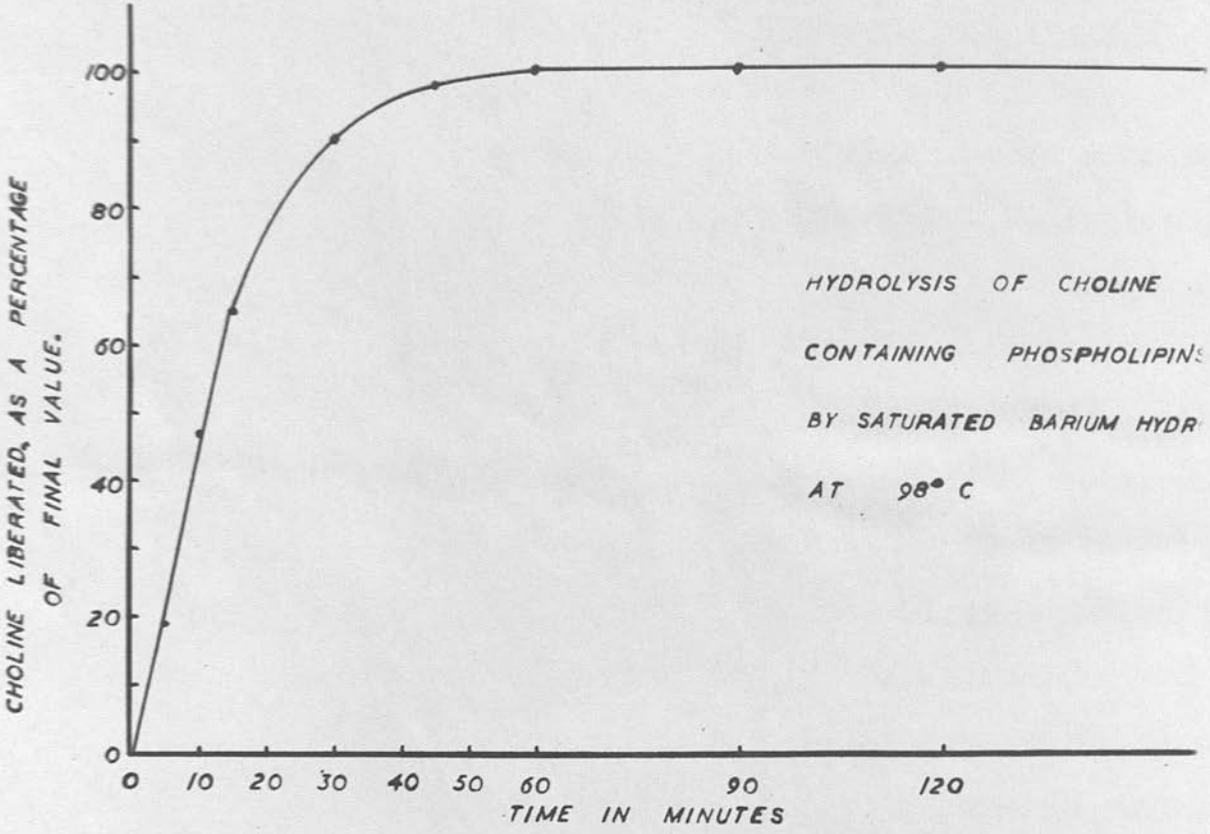

CHOLINE LIBERATED, AS A PERCENTAGE OF FINAL VALUE.
100
80
60
40
20
0
0
10
20
30
40
50
60
90
120
TIME IN MINUTES
HYDROLYSIS OF CHOLINE
CONTAINING PHOSPHOLIPINS
BY SATURATED BARIUM HYDR
AT 98° C

FIGURE 1

immersing in the boiling water bath, duplicate pairs of tubes were analysed for choline as described on page 32.

The hydrolysis curve shown on the opposite page is constructed from the results obtained in this experiment. The graph demonstrates complete liberation of choline in one hour, and also shows that no choline is destroyed even after six hours' hydr-:olysis.

The phospholipins in the same lipoid extract were hydrolysed with N KOH at 37°C for sixteen hours (Hack, 1947) and it was found that the same amount of choline was liberated with this reagent as with the barium hydroxide. As a consequence of these exper-:iments it was decided to employ barium hydroxide as the hydrolytic reagent, and to hydrolyse the lipid extract at 100°C for two hours.

2. Experiment which shows that choline is not adsorbed on barium sulphate.

After the liberation of choline from phospho-:lipins by hot barium hydroxide solution, the hydrol-:ysate must be freed from barium ions (which would interfere with the reineckate precipitation) and the pH/

pH adjusted to 3-4. This is most easily accomplished by the dropwise addition of dilute sulphuric acid. This technique necessitated a control experiment to show that barium sulphate under the conditions pre-:vailing in this experiment, would not adsorb choline.

Into six 15 ml. graduated tubes were placed 1 mg. choline chloride in 5 ml. water and 3 ml. saturated barium hydroxide solution. These tubes were immersed in a boiling water bath for fifteen minutes, one drop of methyl red indicator was then added to each, followed by sulphuric acid dropwise until the indicator just turned permanently red. Neutralisation was commenced with 1.0 N sulphuric acid, 0.1 N acid being employed near the end point. After neutralisation the tubes were removed from the bath, and the solutions diluted to 15 ml., and filtered (No. 42 papers) into short dry boiling tubes. Aliquots of the filtrate (10 ml. equivalent to 0.66 mg. choline chloride) were removed to 8 ml. graduated spectrophotometer tubes, and the precipi-:tation, centrifugation, and spectrophotometry carried out as previously described. The choline chloride estimated by this procedure was identical with/

with that calculated (0.66 mg. per aliquot), showing that no choline had been adsorbed on the barium sulphate.

3. To test the effect of varying the pH on the quantitative precipitation of choline reineckate.

Since different workers have precipitated choline reineckate at widely varying hydrogen ion concentrations, it was necessary to examine the influence of pH on the precipitation. Therefore, a series of M/15 buffers was set up in 8 ml. graduated spectrophotometer tubes in duplicate in the range pH 2-10, the volume of each buffer being 5 ml. To each buffer was added 1 ml. (1 mg.) of standard choline chloride solution followed by 2 ml. saturated aqueous solution of ammonium reineckate. The tubes were placed in the refrigerator for two hours, centrifuged, and the precipitates washed exactly as stated previously, with 0.1 N hydrochloric acid and water. The analysis was completed as before.

From the results shown in Table iii, it seems possible to achieve quantitative precipitation of choline reineckate between pH 2 and pH 10 inclus-:ive; but, since the precipitate was more granular at pH 3-4, for ease in centrifugation it was decided to precipitate the reineckate within this pH range.

Table iii/

Table iii

Precipitation of Choline Reineckate at Varying Hydrogen Ion Concentrations.

pH	2	3	4	5	
Reading at 525 mμ .	0.105 0.110	0.110 0.110	0.110 0.103	0.103 0.108	
mg. Choline Chloride	1.02	1.05	1.02	1.00	
%age "Recovery"	102	105	102	100	
pH	6	7	8	9	10
Reading at 525 mμ .	0.105 0.105	0.103 0.110	0.110 0.113	0.110 0.105	0.100 0.105
mg. Choline Chloride	1.00	1.02	1.05	1.02	0.98
%age "Recovery"	100	102	105	102	98

4. Comparison/

4. Comparison of the colorimetric procedure with the micro-kjeldahl method for the estimation of choline reineckate.

In view of the following experiment in which the colorimetric method was compared with the micro-kjeldahl method, it was decided to adopt the former technique for routine purposes, because the accuracy attainable with it was similar to the more lengthy titrimetric procedure, as may be noted in Table iV.

The colorimetric procedure appeared to give good results when one adhered to the conditions of precipitation as already stated, namely, at pH 4, and leaving the precipitate in the refrigera-:tor for a few hours, provided one used quantities of choline of the order of 0.2-1.0 mg.

Table iv.

Comparison of Micro-kjeldahl and Colorimetric Estimation of Choline Reineckate

(a) Micro-kjeldahl Estimation of Choline Reineckate

Choline Chloride Estimated mg.	Titre 0.012 N Acid	Mean Titre	Choline Chloride Calculated	%age "Recovery" of Choline
0.1	0.422 0.426 0.420	0.423	0.101	101
0.2	0.840 0.850 0.850	0.846	0.202	101
0.4	1.70 1.74 1.76	1.73	0.413	103
1.0	4.30 4.35 4.35	4.33	1.03	103
2.0	8.30 8.35 8.28	8.31	1.99	99

(b) Colorimetric Estimation of Choline Reineckate in Acetone

Choline Chloride Estimated mg.	Optical Density at 525 mμ.	Choline Chloride Calculated	%age "Recovery" of Choline
0.1	.012+	-	-
0.2	.021+	-	-
0.4	.039	0.38	96
1.0	.103	1.01	101
2.0	.195	1.90	95

+ Readings too low to be reliable

5. The/

5. The recovery of choline added to the homogenate.

Two 20 ml. portions of a homogenate of an unincubated egg were placed in separate conical flasks. To one, 10 ml. distilled water was added, and to the other, 20 mg. choline chloride in 10 ml. water. Both flasks were agitated on a mechanical shaker for 15 minutes and duplicate 5 ml. aliquots from each flask were extracted with chloroform-methanol (1:1) in the manner already described, the combined extracts being made up to 100 ml. Duplicate 10 ml. aliquots of these extracts were used in choline determinations, the choline recoveries being tabulated in Table v.

Table v.

Recovery of Choline Added to the Homogenate

Choline Added mg.	Choline Found mg.	Mean Choline Found mg.	Choline Recovered mg.	Choline Recovered as %age
0.0	79 78 79	79	-	-
20.0	100 100 99 99	100	21	105%

This/

This experiment showed that an adequate recovery of added choline could be obtained, and also that the choline estimated by this process would be "total choline" or "combined choline" (as in lecithin) plus "free choline" if such existed. Presumably the free choline was extracted by the organic solvents due to the water which was present in all the homogenates.

EXPERIMENTS TO CHECK THE RELIABILITY OF THE METHIONINE METHOD

1. Studies of certain factors affecting rate of protein hydrolysis.

Before commencing routine estimations of methionine it was essential that certain aspects of protein hydrolysis, such as hydrolysis time and acid concentration, should be studied, and towards this end a bulk sample of mixed egg protein was prepared. A homogenate prepared from three eggs was extracted with 600 ml. chloroform-methanol (1:1) on a steam bath for eight hours, and the lipoid extract decanted off. This refluxing was repeated for a further four hours using 600 ml. ethanol-ether (3:1) and the/

the resulting granular white product was sucked dry on a Buchner funnel for half an hour. This mixed protein was ground to a fine powder in a mortar and dried over phosphorus pentoxide for several days, finally yielding about 11 g. protein as an amorphous white powder.

(a) Rate of hydrolysis with "constant-boiling" hydrochloric acid.

About 200-300 mg. protein was accurately weighed into each of twelve boiling tubes fitted with B19 sockets. Constant-boiling hydrochloric acid (5 ml., 5.5N) was added to each tube together with a small heavy glass cup to prevent bumping. (This cup was fitted with a long glass stem to keep it inverted like a diving bell.) The tubes, fitted with reflux condensers and immersed in a bath of dibutylphthalate at 125°C, were shaken occasionally to assist solution of the protein. The tubes were removed from the bath in pairs, after 1, 2, 4, 8, 12 and 30 hours' hydrolysis. The hydrolysates were diluted with 5 ml. water and 100 mg. activated charcoal ("Norit") was added to each. The loosely stoppered tubes were left in a boiling water bath for/

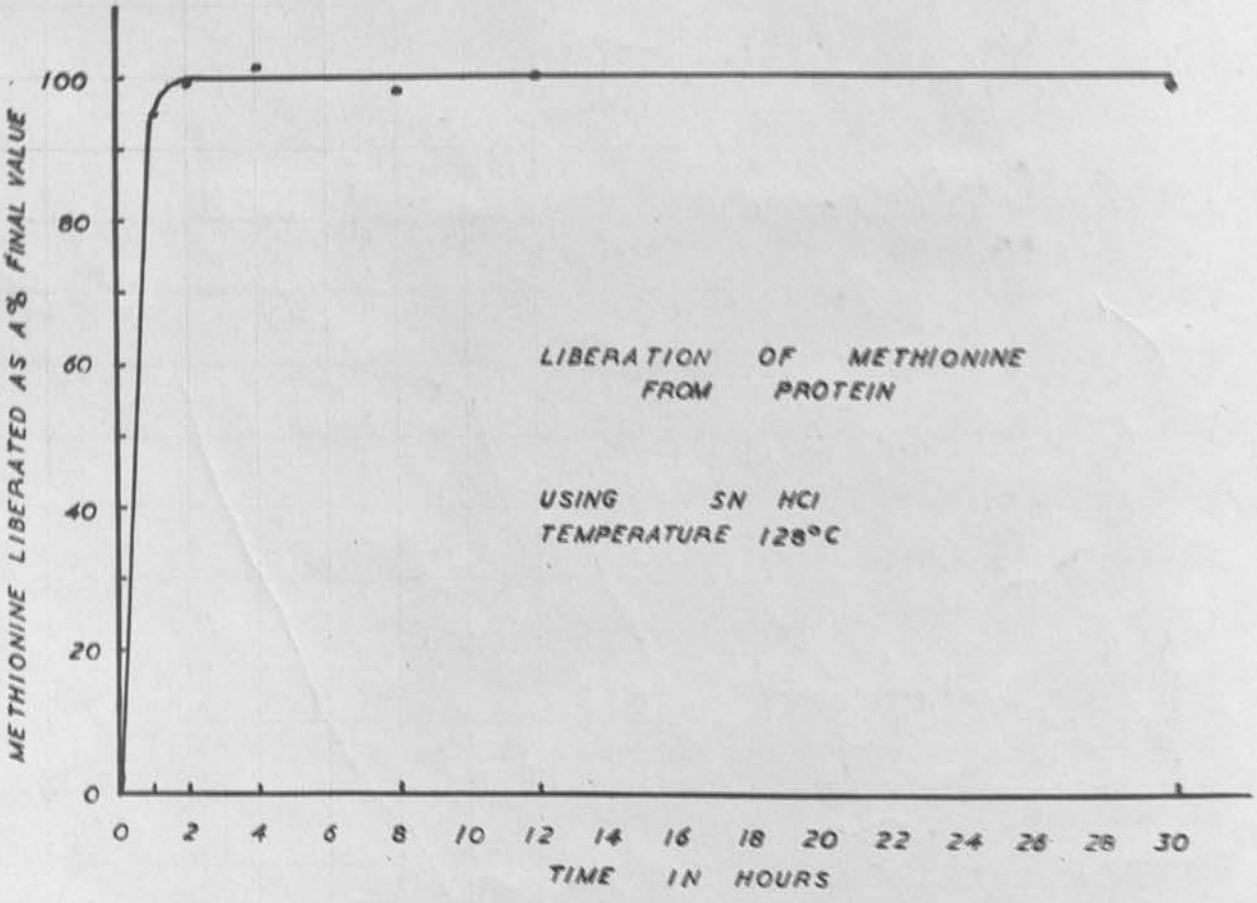

METHIONINE LIBERATED AS A % FINAL VALUE
100
80
60
40
20
0
LIBERATION OF METHIONINE
FROM PROTEIN
USING 5N HCl
TEMPERATURE 128°C
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME IN HOURS

FIGURE ii

for two hours. Tubes 1, 2 and 3 (that is, up to and including 4 hours' hydrolysis) required at least four hours to complete the decolourisation, and on occasions, even longer. It was necessary to activate the "Norit" by heating it in a silica basin over a Meker burner, and to store it in a desiccator. When all the hydrolysates were colourless, they were filtered through micro-Hirsch funnels and the estim-:ation of the methionine was completed as described on page 34. Using the McCarthy and Sullivan (1941) technique with the modifications outlined, the rate of liberation of methionine from the protein is shown in Figure ii on the opposite page. This graph shows almost complete liberation of methionine in one hour, with no destruction even after 30 hours' hydrolysis. As already noted, the humin produced during the hydrolysis was difficult to remove unless the reaction had been in progress for over four hours, and so for routine purposes a hydrolysis time of six hours was adopted.

Since sodium chloride affected the colour reaction, it seemed highly desirable to hydrolyse the protein/

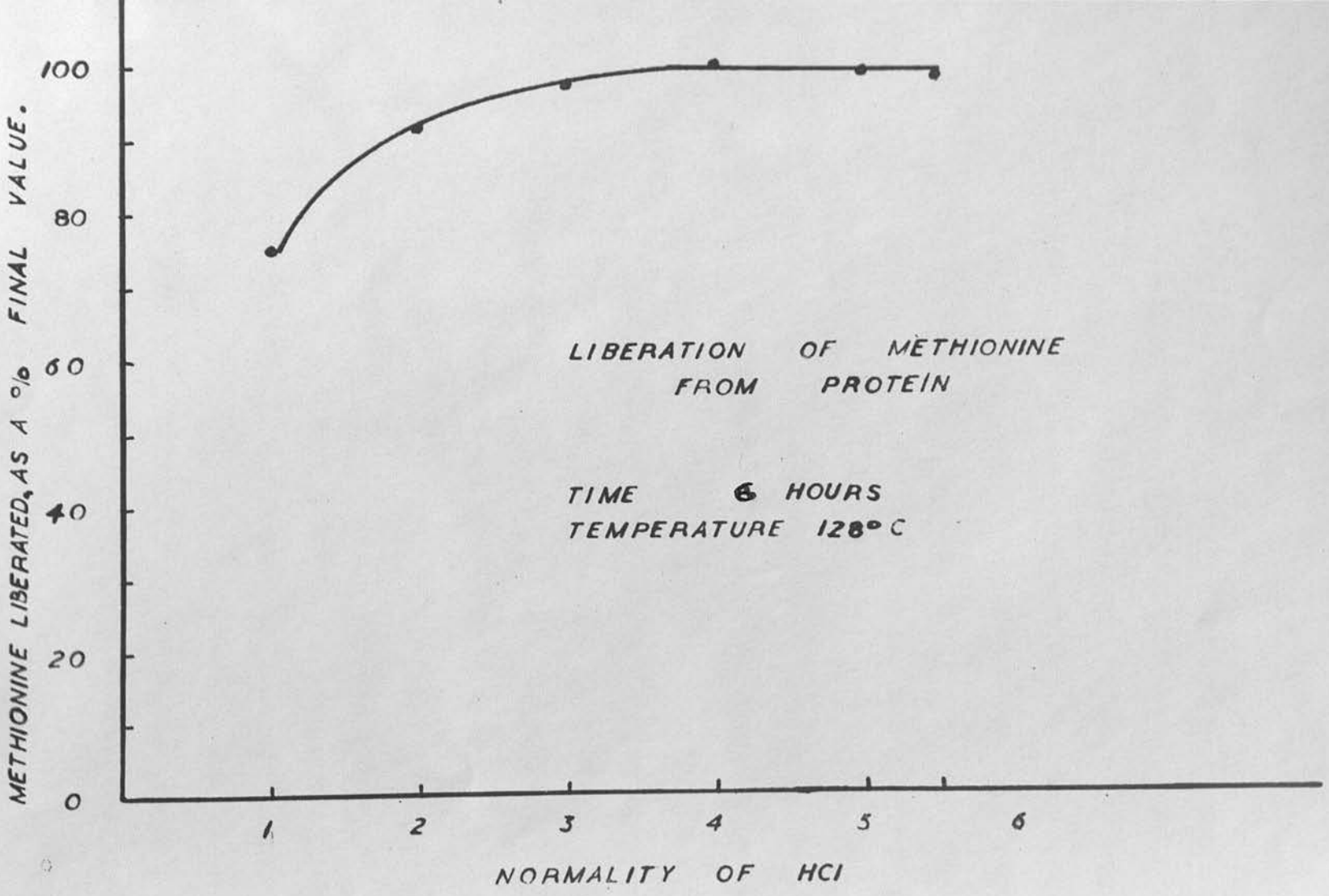

METHIONINE LIBERATED, AS A % FINAL VALUE.
100
80
60
40
20
0
1
2
3
4
5
6
NORMALITY OF HCl
LIBERATION OF METHIONINE
FROM PROTEIN
TIME 6 HOURS
TEMPERATURE 128° C

FIGURE iii

protein with the minimum concentration and volume of acid, even although the sodium chloride effect was being compensated in the calibration graph.

(b) The effect of acid concentration on the liberation of methionine from protein during 6 hours' hydrolysis at 125°C.

Using the previously acquired knowledge of optimum time for hydrolysis, an experiment was con-:ducted along the lines of the last one described. The hydrolysis time was fixed, while the acid concentration was varied from 1.0-5.5 N hydrochloric acid, the temperature being maintained at 125°C and the volume of acid 5 ml. After hydrolysis, the methionine content of each solution was estimated. The results are plotted graphically in Figure iii which shows the maximum amount of methionine to be liberated by 4N hydrochloric acid in 6 hours.

A repeated hydrolysis/time experiment with this concentration of acid showed complete liberation of methionine in 6 hours and the hydrolysates could be decolourised quite easily with charcoal ("Norit").

2. Interference from other amino acids.

McCarthy and Sullivan (1941) reported interference from two amino acids, tryptophane and histidine. The former is of little importance because it is destroyed during acid hydrolysis as was demonstrated in this work by a negative Hopkins-Cole test in 140 consecutive methionine determinations. The original authors found that the addition of 1% glycine suppressed the colour produced by nitro-:prusside and histidine, and in practice, this artifice was found to be most effective. McCarthy and Sullivan stated that a mixture of fifteen amino acids other than methionine and tryptophane gave a negative test for "methionine" in this colour reac-:tion, and upon addition of methionine to this mixture, it was possible to attain a 99.6% recovery. It was decided in this work to test each amino acid separately, in case possible colour enhancement produced by one amino acid was being fortuitously suppressed by another. The results of this invest-:igation are shown in Table vi. Only one amino acid, tryptophane, interfered, and of course it would not be present in an amino acid hydrolysate, using hydrochloric acid to hydrolyse the protein.

Table vi/

Table vi.

Amino acid	Amount taken mg.	Methionine equivalence using the colorimetric method
Leucine	5	0.00
Isoleucine	10	.01
Valine	5	.02
Serine	9	.01
Threonine	9	.02
Ornithine	9	.00
Lysine	7	.00
Arginine	12	.00
Tryptophan	9	2.96
Histidine	5	0.05
Cysteine	11	.03
Cystine	10	.00
Aspartic acid	5	.00
Glutamic acid	5	.00
Proline	6	.00
Hydroxy proline	7	.01
Phenylalanine	7	.01
Alanine	9	.00
Tyrosine	6	.01

N.B. The 'Methionine Equivalence' is the optical density of the solution expressed as methionine using the Calibration Graph (Figure 3)

3. The/

3. The adsorption of methionine on "Norit" and the recovery of methionine added to the protein.

Standard solutions of methionine (10 ml.) of various concentrations in 4N hydrochloric acid, were treated with 100 mg. "Norit", heated on a boiling water bath for fifteen minutes, and filtered through a micro-Hirsch funnel. Aliquots of the filtrate were transferred to 25 ml. volumetric flasks, neutralised and the methionine estimated as previously described. This experiment showed that methionine was adsorbed on activated charcoal in the presence of acid (see Table vii). It seemed possible, however, that methionine would not be adsorbed in the presence of other amino acids. Also, since the filter containing the "Norit" was washed with 0.1 N hydrochloric acid in practice, it was considered that this procedure might elute some of the adsorbed methionine.

In order to test the first point, about a gram of protein was accurately weighed out and hydrolysed for 6 hours with 4N hydrochloric acid at 125°C. The hydrolysate was transferred to a 100 ml. graduated flask, made up to volume with distilled water, and well shaken. Four 20 ml. portions of this brown-black solution were removed to boiling tubes./

tubes. Two of the tubes were treated with a solu-:tion of 5 mg. methionine in 5 ml. water, while the remaining two tubes received 5 ml. water each. All four tubes then received 100 mg. "Norit", and were left on a boiling water bath for two hours. The hydrolysates were then filtered, the residue on the filter washed with 0.1 N hydrochloric acid, and the filtrates neutralised, made up to 25 ml., and the methionine content assayed in the usual way. The recovery of added methionine (102%) showed that the added methionine was not adsorbed, and indeed, that all stages of the analysis were satisfactory. Criticism of this experiment could be made on the grounds that perhaps "Norit" adsorbed a small but constant amount of methionine from each solution, and was then satiated, with the result that the "exogenous" methionine would not be adsorbed. A synthetic hydrolysate consisting of nineteen amino acids was therefore, prepared, and a fixed amount of methionine added to the solution. The mixture was made up to 100 ml. with 4N hydrochloric acid and the methionine content of the solution was estimated. Aliquots (10 ml.) were treated with 100 mg. "Norit" and left on a boiling water bath for two hours in loosely stoppered tubes. This

"hydrolysate"/

"hydrolysate" was then filtered, and the "Norit" washed with 0.1 N hydrochloric acid. The filtrate was neutralised, made up to 25 ml. and methionine estimated on an aliquot. From the results it was apparent that methionine was not adsorbed on "Norit" in the presence of other amino acids. This is in general agreement with the well known fact that aromatic amino acids are preferentially adsorbed on activated carbon.

Table vii summarizes these experiments.

Table vii

Adsorption of Methionine on "Norit"

Experiment	Methionine Added	Methionine Recovered	%age Recovery
	mg.	mg.	%
Methionine Solution + "Norit"	2.50	1.97	79
Protein Hydrolysate + Methionine, + "Norit"	3.33	3.40	102
Synthetic Amino Acid Mixture, + Methionine, + "Norit"	5.00	5.08	102

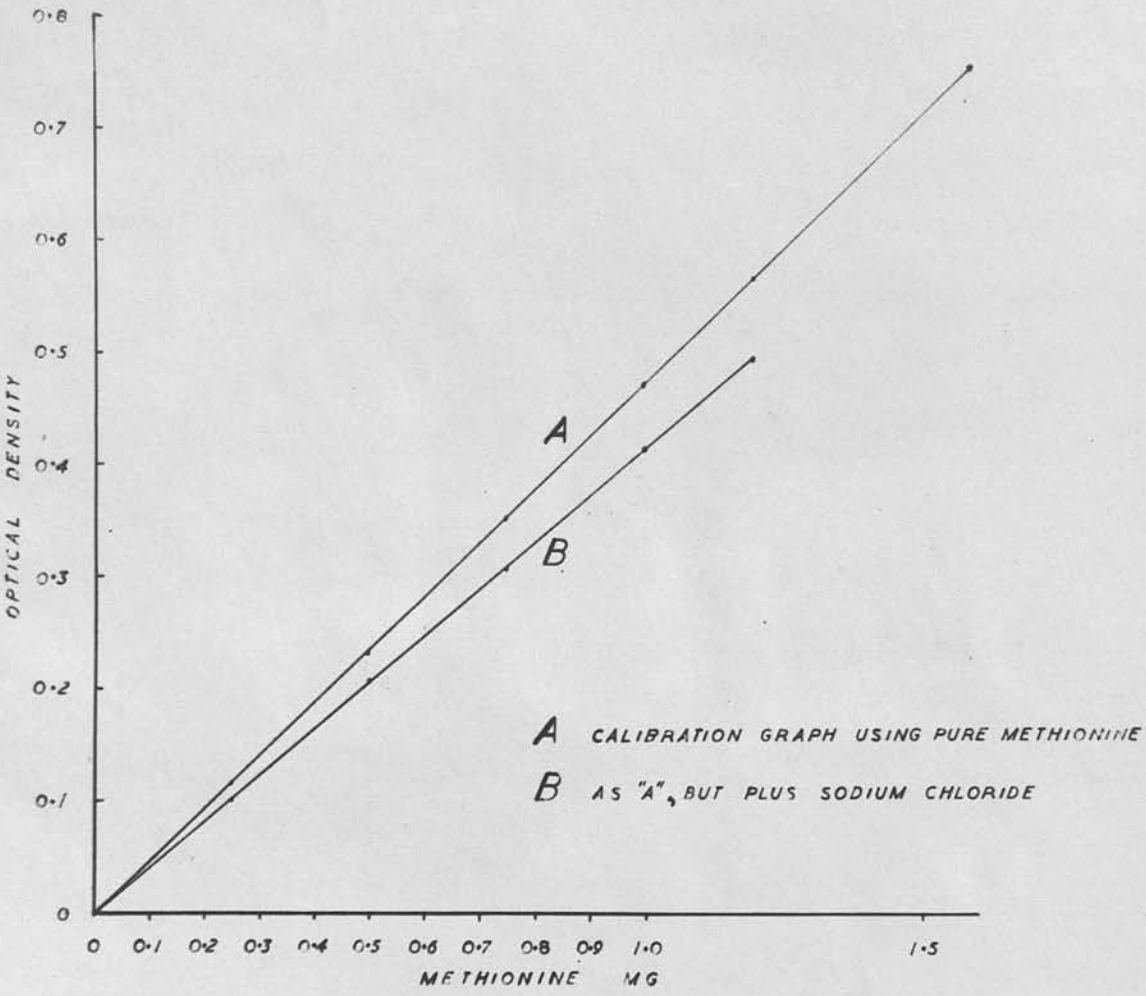

0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
OPTICAL DENSITY
A
B
A CALIBRATION GRAPH USING PURE METHIONINE
B AS "A", BUT PLUS SODIUM CHLORIDE
0 0·1 0·2 0·3 0·4 0·5 0·6 0·7 0·8 0·9 1·0 1·5
METHIONINE MG

FIGURE IV

4. Factors affecting the colour reaction, and hence the calibration graph.

It appeared highly desirable to ascertain whether sodium chloride produced during the neutralisation of the hydrolysate would have any effect on the colour reaction. Sodium chloride (2 g.) was added to 50 mg. methionine and the whole made up to 50 ml. with water. This solution represented the hydrolysis of a protein with 6 ml. 6 N hydrochloric acid, followed by neutralisation with sodium hydroxide, and the mixture made up to 50 ml. This methionine standard solution was used to construct a new calibration graph; when this graph was compared with one prepared from methionine alone, it was found that sodium chloride depressed the colour produced in the reaction. This fact appears to have escaped the notice of other workers who used this method. The calibration graphs are shown in Figure iv opposite. In practice, the calibration graph was always constructed with pure methionine dissolved in the appropriate amount of sodium chloride solution.

EXPERIMENTS/

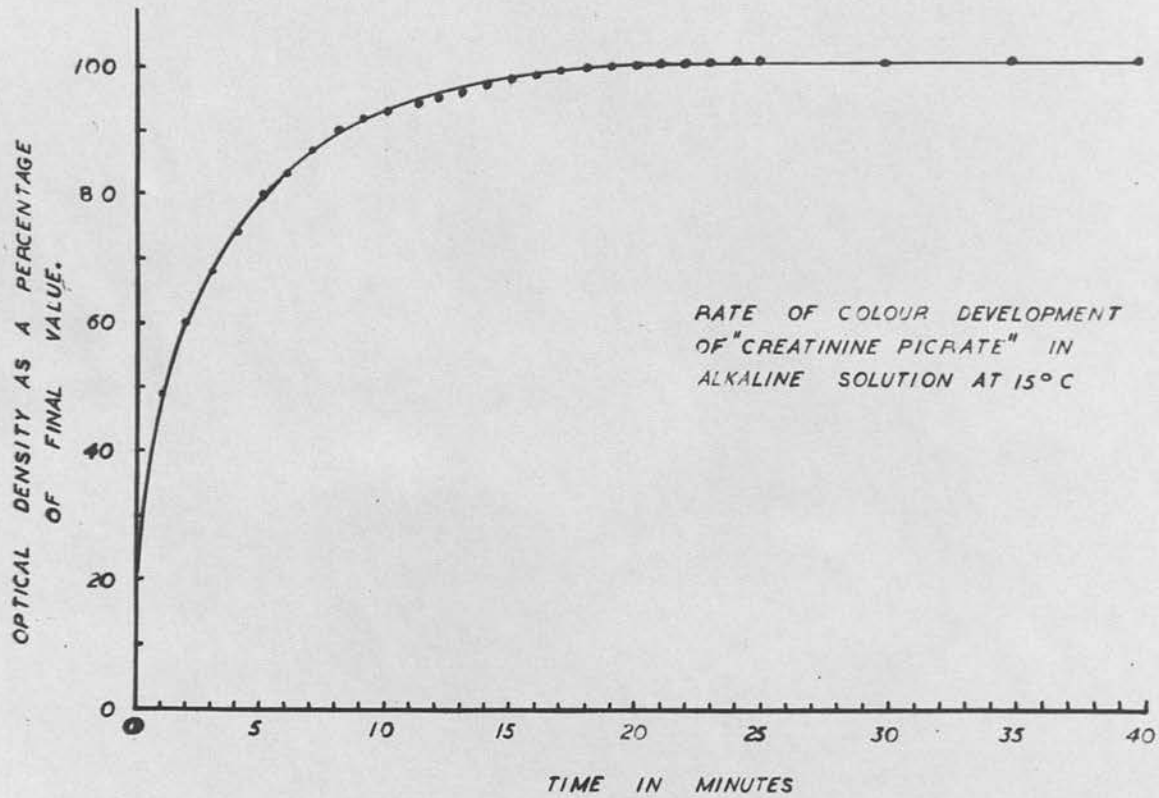

OPTICAL DENSITY AS A PERCENTAGE OF FINAL VALUE.
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
TIME IN MINUTES
RATE OF COLOUR DEVELOPMENT OF "CREATININE PICRATE" IN ALKALINE SOLUTION AT 15° C

FIGURE V

EXPERIMENTS TO CHECK THE RELIABILITY OF THE CREATINE AND CREATININE PROCEDURES

1. The rate of colour development of "creatinine picrate".

Creatinine (50 µg.) in 5 ml. was transferred to a 20 ml. graduated tube and 5 ml. alkaline picrate (see Appendix II) added, and the mixture made up to volume with water. Readings of the optical density of this solution were made against a reagent blank at 1 minute intervals in the diffraction grating spectrophotometer set at 510 mµ, a wavelength found to be close to the absorption maximum of the colour. The results of this experiment are depicted in Figure V, which shows that the colour reaction attains a steady state after twenty minutes at room temperature. It was therefore decided to read the colour absorption in the spectrophotometer twenty minutes after the addition of the alkaline picrate.

2. The rate of conversion of creatine to creatinine at various temperatures.

Creatine (50 µg.) in 5 ml. solution was delivered into tubes graduated at 20 ml., followed by 10 ml. HCl-KCl buffer (0.1N, pH 1.2). These tubes/

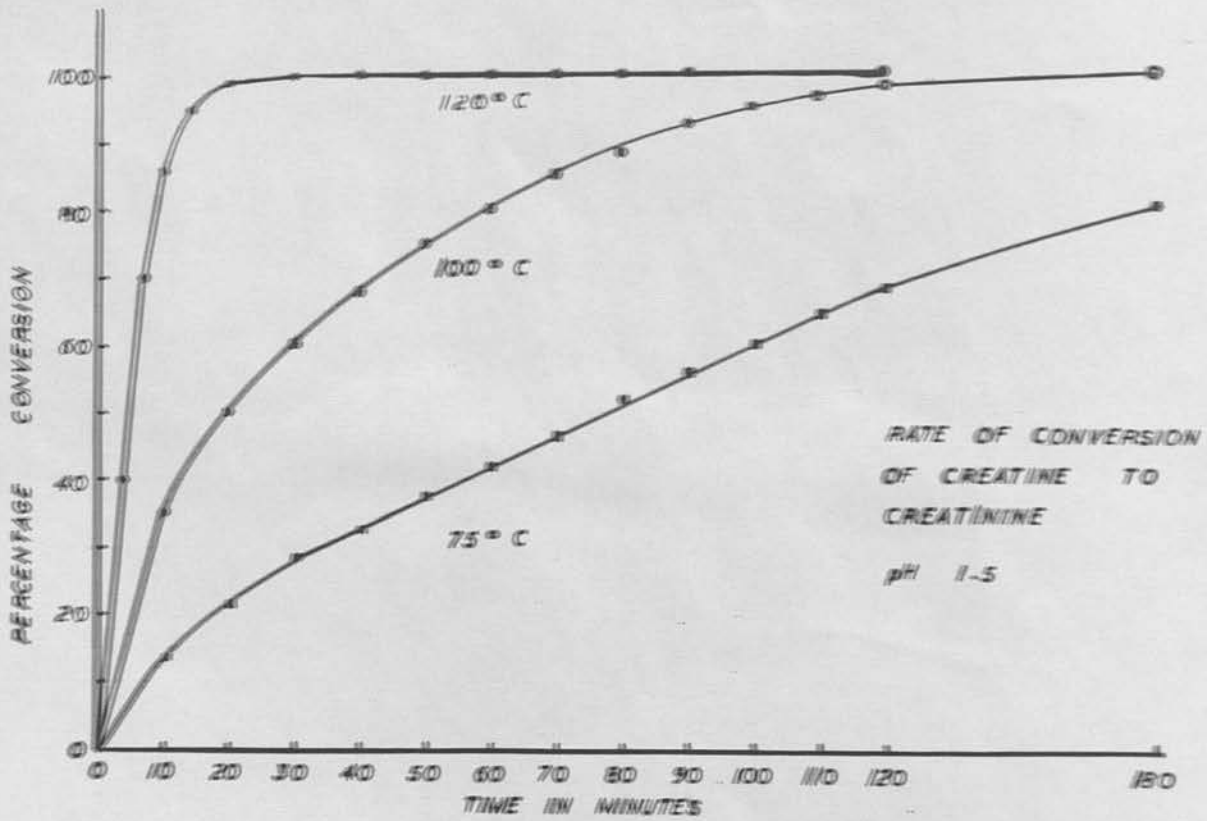

PERCENTAGE CONVERSION
100
80
60
40
20
0
120° C
100° C
75° C
RATE OF CONVERSION
OF CREATINE TO
CREATININE
pH 1-5
0 10 20 30 40 50 60 70 80 90 100 110 120 130
TIME IN MINUTES

FIGURE Vi

tubes were simultaneously placed in a water bath at 100°C and removed at intervals up to three hours. Upon removal of each tube from the water bath, the solution was adjusted to pH 7 and stored in the refrigerator. The creatinine content of each solution was then estimated by the picrate method previously described.

This experiment was repeated twice, once at an elevated temperature (120°C in the autoclave), and also at a lower temperature (75°C). The results obtained in these three experiments are shown graphically in Figure vi on the opposite page.

3. The rate of conversion of creatine to creatinine at various hydrogen ion concentrations.

As a consequence of the last experiment, it was decided to study the rate of conversion of creatine to creatinine, varying the pH of the creatine solution, and fixing the temperature at 120°C and the time of heating at 30 minutes.

The results of this investigation, which was conducted in a similar manner to the last experiment, are shown in Figure vii. The graph shows complete conversion of creatine to creatinine at pH 1.

In/

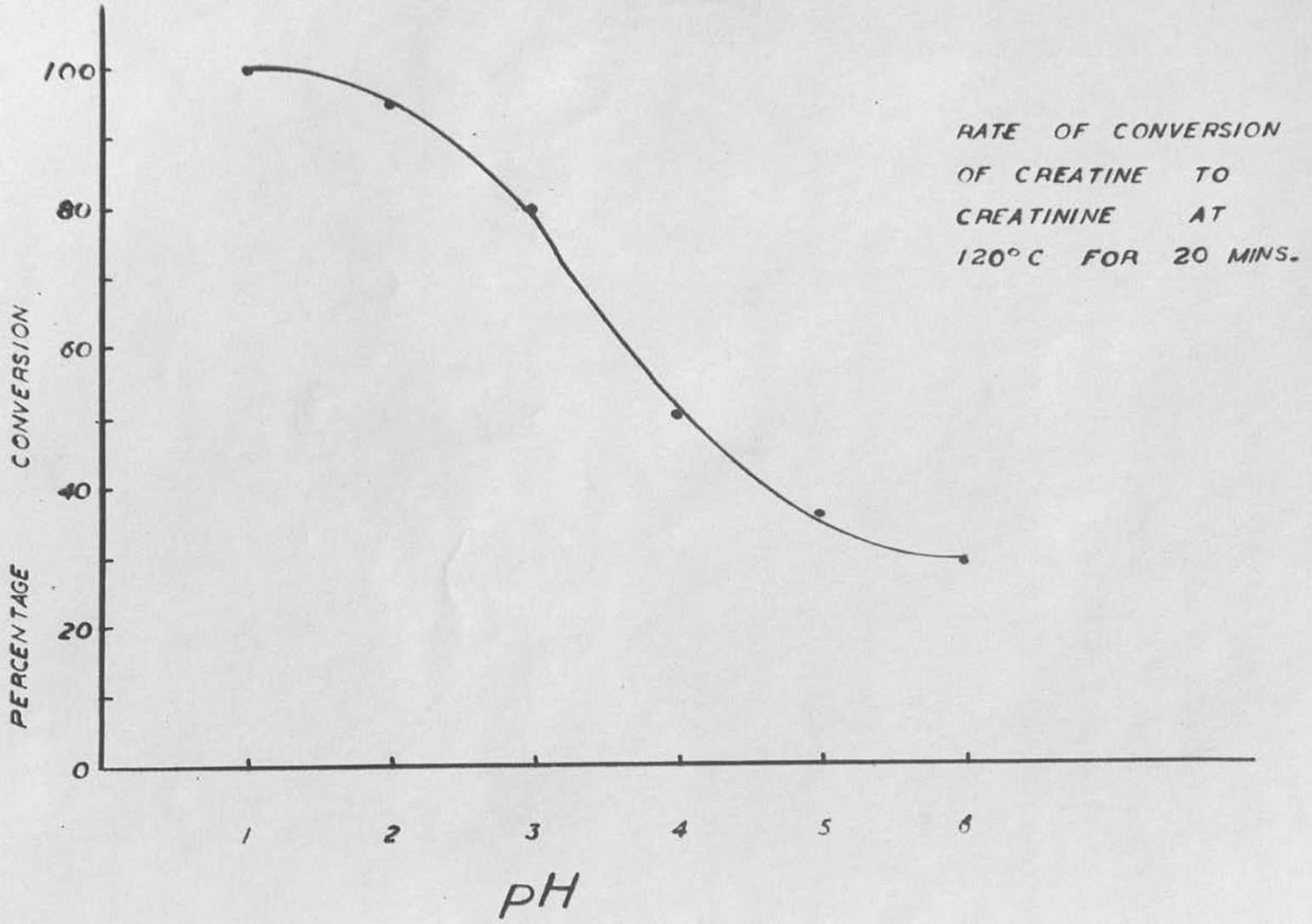

RATE OF CONVERSION OF CREATINE TO CREATININE AT 120° C FOR 20 MINS.
PERCENTAGE CONVERSION
100
80
60
40
20
0
1
2
3
4
5
6
pH

FIGURE VII

In view of these results, the conditions decided upon for the quantitative conversion of creatine to creatinine were as follows: adjustment of the protein-free filtrate containing creatine approximately to pH 1, and heating at 120°C for 30 minutes.

After this work was completed, Clark and Thompson (1949) published experimental results almost identical with those recorded here.

4. Creatine and creatinine recovery experiment.

Aliquots (20 ml.) of an unincubated egg homogenate were delivered into four conical flasks, and to each homogenate was added one of the following solutions.

(1) 10 ml. water.

(2) 10 ml. creatinine solution (450 µg.).

(3) 10 ml. creatine solution (225 µg.).

(4) 5 ml. creatine solution (210 µg.) and 5 ml. creatinine solution (225 µg.).

The/

The flasks were agitated in a mechanical shaker for 15 minutes, 30 ml. tungstic acid was added to each, and the mixture filtered. The pH of the filtrate (about 1) was suitable for the conversion of creatine to creatinine. Duplicate 5 ml. aliquots of each filtrate were pipetted into 20 ml. graduated tubes; one tube was capped with lead foil and autoclaved as previously described in order to convert creatine to creatinine. The creatinine content of each solution was estimated in the usual way, and the percentage recovery of the added bases calculated.

These results which are reported in Table viii show that a complete recovery of added creatine and creatinine could be accomplished within the experimental limits of the estimation.

Table viii

Recovery of Creatine and Creatinine added to an Egg Homogenate.

Flask.	Creatine added µg.	Creatinine added µg.	Total Creatinine + Found µg.	Preformed Creatinine + Found µg.	%	Creatine + Found µg.	%
1.	-	-	-	-	-	-	-
2.	-	450	468	468	104	-	-
3.	420	-	360	-	-	416	99
4.	210	225	408	216	96	222	106

+ Preformed creatinine = creatinine before autoclave treatment.

Total creatinine = " after " "

Creatine = (Total creatinine - preformed creatinine) X 1.16

APPENDIX II

(a) Technique Employed in Purification of Solvents

Ethanol

Ethanol (D.C.L., A.R. grade) was refluxed with solid sodium hydroxide pellets for eight hours, using 100 g. alkali per litre of alcohol. The dark brown liquid was distilled into a flask containing freshly prepared quicklime, and the distillate allowed to remain in contact with this reagent for 24 hours. The alcohol was redistilled directly into the winchester in which it was to be stored.

Methanol

The procedure was exactly the same as for the purifi cation of ethanol.

Butanol

This alcohol was refluxed with sodium hydroxide in the same manner as ethanol, but the drying proced-:ure with quicklime was omitted.

Chloroform

The purchased liquid, of A.R. grade, was washed with half its volume of water in a separating funnel; the chloroform layer was then dried with calcium chloride, and redistilled twice.

Ether/

Ether

Ether (A.R. grade) was washed with half its volume of 5% ferrous sulphate solution, and the ethereal phase dried with calcium chloride for 24 hours. The liquid was then redistilled twice, and stored in a dark bottle; this procedure yielded a peroxide-free reagent, as tested with V_2O_5 in concentrated sulphuric acid.

Acetone

Acetone of B.P. quality, was refluxed for eight hours with potassium permanganate (10 g./litre), distilled into a flask containing quicklime, and redistilled from this reagent.

Glacial Acetic Acid

Glacial acetic acid (A.R. grade) was refluxed for 4 hours with potassium dichromate (5 g./litre) and distilled directly into a storage winchester.

(b) Purification of Certain Reagents.

Choline Chloride

The supplied choline chloride (B.D.H) was twice recrystallised from absolute ethanol, and dried at 60°C in vacuo for 12 hours. This substance gave on analysis:

	% Nitrogen	% Chlorine
Theoretical:	10.04	25.4
Found:	9.90	25.9

Betaine Hydrochloride

Betaine hydrochloride (B.D.H.) was twice re-:crystallised from water, and dried at 60°C *in vacuo* for 12 hours.

Ammonium Reineckate

The supplied salt (B.D.H.) was recrystallised from water at 70°C and dried in a vacuum desiccator over calcium chloride.

Sodium Nitroprusside

Commercial grade sodium nitroprusside was re-:crystallised three times from water at 70°C, and dried in a vacuum desiccator over calcium chloride.

Picric Acid

The supplied acid was of commercial grade, and was recrystallised twice from glacial acetic acid, and carefully dried in a vacuum desiccator over calcium chloride.

Methionine

This amino acid (L. Light) was recrystallised from water and dried at 60°C *in vacuo* for 24 hours. The substance gave on analysis:

	% Nitrogen	% Sulphur
Theoretical:	9.4	21.5
Found:	8.95	21.2

Creatine and Creatinine

Both these substances were twice recrystallised from water and dried at 60°C *in vacuo* for 12 hours. On analysis, they gave:

		% Nitrogen
Creatine:		
	Theoretical:	32.08
	Found:	32.01
Creatinine:		
	Theoretical:	37.2
	Found:	36.16

(c) The Preparation of Monomethylaminoethanol, and dimethylaminoethanol.

The first base was prepared by the condensation of methylamine and ethylene oxide, and the second by the condensation of dimethylamine and ethylene oxide, according to the method of Matthes (1901). These substances were purified by distillation *in vacuo*, and the melting points of the picrates of these bases agreed with the values in the literature.

APPENDIX III.

A. Tables of analytical results.
choline hydroxide.

Approximate Incubation Period. (days)	Embryo Weight. (g.)	Total choline hydroxide in System. (mg.)	Choline hydroxide in Embryo (mg.)
0	0	181 188 184 201 191 177 196 186 170 153 165 170 160 193 202 183	0
6	0.4 0.5 0.4 0.4 0.6 0.4 0.4 0.5 0.5	178 190 180 170 162 168 180 148 183	all less than 0.3 mg.
9	2.5 2.7 2.6 1.9 2.0 2.0	170 180 166 161 169 168	2.0 2.1 2.0 2.2 2.2 2.1

Choline hydroxide results continued.

Approximate Incubation Period. (days)	Embryo Weight. (g.)	Total Choline hydroxide in System. (mg.)	Choline hydroxide in Embryo. (mg.)
10	2.9	168	1.8
	3.1	179	2.1
	2.8	175	1.8
	3.1	187	2.0
	2.9	180	2.0
	3.4	170	2.0
	3.0	173	2.0
	3.2	181	2.2
12	5.8	162	2.9
	4.9	173	2.7
	6.7	158	3.2
	5.8	162	2.8
	7.1	178	2.8
	6.6	173	4.1
	6.8	166	3.9
	6.0	170	3.6
13	9.5	177	5.3
	9.4	161	5.5
	9.1	171	4.8
	9.1	159	4.6
	8.7	151	4.7
	10.4	169	5.1
	9.7	165	4.9
14	12.0	180	7.8
	12.9	171	8.3
	13.4	146	6.9
	11.8	161	7.4
	11.9	157	7.1
	12.3	152	8.0

Choline hydroxide results continued.

Approximate Incubation Period (days.)	Embryo weight. (g.)	Total Choline hydroxide in System (mg.)	Choline hydroxide in Embryo (mg.)
15	15.9	169	11.7
	15.3	157	11.2
	15.8	149	10.1
	16.0	153	10.7
	14.8	147	10.9
	15.1	160	11.1
17	20.0	139	16.0
	19.3	129	18.0
	21.1	127	18.1
	20.6	160	20.1
	19.2	158	19.0
	21.0	149	21.9
	20.7	139	18.9
	20.3	133	19.3
18	24.7	146	23.0
	27.0	141	28.1
	24.7	140	24.5
	26.1	129	26.3
	25.8	127	26.8
	24.9	125	24.2
	24.0	127	26.1
	23.8	128	25.4
20	34.4	105	43.1
	34.3	112	35.4
	34.1	101	37.6
	33.8	80	43.2
	34.4	90	40.0
	33.9	93	45.0
21	35.1	88	59.3
	34.8	78	51.4
	35.6	98	56.2
	34.0	97	54.7
	36.1	97	56.2
	35.8	98	55.8

B. Tables of Analytical Results.
Methionine.

Approximate Incubation Period. (days)	Embryo Weight. (g.)	Total Methionine in System. (mg.)	Methionine in Embryo. (mg.)
0	0	181 169 161 151 166 164 157 178 152 177 169 159 168 166	0
6	0.4 0.4 0.5 0.5 0.5 0.6 0.6 0.5 0.6 0.6	160 168 169 164 166 168 171 170 174 161	all less than 0.7 mg.
9	1.9 2.0 2.0 2.5 2.5 2.6	173 180 167 161 172 155	1.5 1.6 1.6 3.1 3.4 3.2

Methionine Analytical Results continued.

Approximate Incubation Period. (days)	Embryo Weight. (g.)	Total Methionine in System. (mg.)	Methionine in Embryo. (mg.)
10	2.9	177	2.3
	3.1	174	2.3
	2.8	142	2.3
	3.1	163	2.2
	2.9	155	3.0
	3.4	158	3.1
	2.9	167	2.8
	3.1	168	2.7
12	6.0	160	8.2
	6.8	149	9.7
	6.6	156	9.2
	7.1	174	7.5
	5.8	157	6.7
	6.7	168	6.8
	7.0	176	8.4
13	9.4	167	11.4
	9.1	153	13.0
	9.1	149	11.9
	8.7	158	8.5
	10.4	164	10.7
	9.5	170	8.7
15	15.0	170	23.0
	14.8	162	24.7
	14.0	164	22.1
	14.7	153	25.2
	14.1	158	22.6
	13.9	179	23.4

Methionine Analytical Results continued.

Approximate Incubation Period (days)	Embryo Weight. (g.)	Total Methionine in System. (mg.)	Methionine in Embryo. (mg.)
17	20.7	168	35.0
	20.6	176	39.4
	19.2	145	35.5
	20.0	135	50.0
	19.3	170	50.1
	21.1	139	46.0
	19.3	164	34.6
19	30.9	159	76.2
	27.4	133	68.1
	28.3	158	64.4
	30.0	147	73.3
	28.7	160	70.5
	28.2	163	68.0
	27.6	143	65.0
	27.9	149	62.0
	28.3	139	64.1
20	34.3	159	77.1
	34.4	151	84.3
	35.3	160	90.2
	35.7	138	83.8
	32.9	129	87.5
	33.8	147	82.3

C. Tables of Analytical Results.
Creatine.

Approximate Incubation Period. (days)	Embryo Weight. (g.)	Total Creatine in System. (mg.)	Creatine in Embryo. (mg.)
8	1.2	0.21	0.20
	1.2	0.25	0.20
	1.1	0.18	0.16
	1.0	0.24	0.23
	1.2	0.23	0.22
	1.3	0.20	0.20
10	3.1	0.45	0.40
	3.0	0.67	0.62
	2.8	0.43	0.40
	2.7	0.49	0.38
	2.9	0.39	0.31
	3.1	0.43	0.40
	3.0	0.51	0.45
	3.2	0.48	0.40
12	6.7	1.23	1.12
	5.8	1.47	1.33
	7.1	1.48	1.20
	6.6	1.32	1.11
	6.8	1.41	1.34
	6.0	1.42	1.40
14	12.0	4.9	4.6
	12.2	4.6	4.3
	11.7	5.1	5.0
	11.3	5.0	4.9
	12.1	5.3	5.0
	12.4	4.8	4.6

Creatine Analytical Results continued.

Approximate Incubation Period. (days)	Embryo Weight. (g.)	Total Creatine in System. (mg.)	Creatine in Embryo. (mg.)
15	15.0	5.4	5.2
	15.3	5.6	5.3
	16.0	5.7	5.4
	14.8	4.7	4.6
	14.7	5.1	5.0
	14.3	5.3	5.1
16	17.1	8.1	7.6
	16.8	8.0	7.5
	16.9	7.9	7.4
	16.9	7.7	7.4
	17.3	8.1	7.8
	17.7	8.0	7.4
17	20.0	11.3	10.7
	19.3	10.5	9.7
	21.1	11.3	10.6
	20.6	10.8	10.1
	19.2	9.7	9.4
	21.0	9.4	9.2
	20.7	10.5	10.0
18	24.7	14.6	13.9
	26.1	15.1	14.3
	25.8	14.8	14.4
	24.9	13.9	13.4
	24.0	14.7	14.0

Creatine Analytical Results continued.

Approximate Incubation Period. (days)	Embryo Weight. (g.)	Total Creatine in System. (mg.)	Creatine in Embryo. (mg.)
19	28.3	21.0	20.0
	30.0	20.7	20.1
	28.7	21.3	19.9
	28.2	20.9	19.8
	27.6	18.9	18.3
	27.9	21.9	21.0
	28.3	21.0	20.0
20	34.3	28.1	26.0
	34.4	25.0	23.6
	35.3	24.1	22.1
	35.7	25.9	24.3
	32.9	26.0	23.8
21	34.8	30.1	26.1
	35.8	30.4	26.7
	35.6	29.6	26.3
	34.0	27.9	24.1
	36.1	29.2	27.4
	35.1	31.0	28.0

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conditions. The amylase activities of the medium and tissue were measured by the method of Smith & Roe (1949). By this procedure synthesis as well as secretion of amylase could be studied.

A progressive increase in 'total amylase activity' (sum of activities of tissue and medium) was observed when slices were incubated aerobically in saline media containing glucose. A greater increase occurred in serum containing added glucose. The increase was greatest (over 100% in some experiments after 2 hr. incubation) when a mixture of amino-acids was added (see Table 1). There was no increase in total amylase activity under anaerobic conditions, or in the presence of 2:4-dinitrophenol (10^{-4}M), cyanide (10^{-4}M) or iodoacetate (10^{-4}M). These observations indicate that the formation of amylase depended on a supply of energy and suggest that the increase in amylase activity represented a true synthesis of enzyme and not an activation of a closely related precursor.

In the presence of cholinergic drugs the fraction of amylase in the medium was significantly increased. This increase did not occur anaerobically or in the presence of 2:4-dinitrophenol (10^{-4}M) or cyanide (10^{-4}M). Thus pigeon pancreas slices appear to be capable of actively secreting amylase *in vitro*. Under the conditions of these experiments synthesis of amylase was not appreciably affected by secretion. *In vitro* secretion of an enzyme (pepsin) has also been reported by Edwards & Edwards (1949).

Table 1. *Synthesis and secretion of amylase by pigeon pancreas slices* in vitro

(2 hr. aerobic incubation. 0·2% glucose.)

Exp. no.	Conditions	Amylase activity (units of Smith & Roe mg. initial dry wt.) Medium	Tissue	Total
1	Unincubated slice	—	59	59
	Incubated in bicarbonate saline	27	40	67
	Incubated in inactivated sheep serum	26	67	93
	Incubated in bicarbonate saline with 0·4% tryptophan-supplemented acid casein hydrolysate	31	75	106
2	Unincubated slice	—	64	64
	Incubated in bicarbonate saline	28	78	106
	Incubated in bicarbonate saline with mixture of 21 amino-acids	32	115	147
	Incubated in bicarbonate saline with 0·4% tryptophan-supplemented acid casein hydrolysate	30	113	143

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Choline and Methionine in the Embryonated Hen Egg. By G. S. Boyd. (*Department of Biochemistry, University of Edinburgh*)

Seventy eggs incubated for periods between 0 and 21 days have been dissected after freezing in acetone-CO_2. Homogenized embryos and 'rests' ('rest' = yolk + white + allantoic and amniotic fluids + membranes) have been separately analysed for choline (method modified from Ramsay & Stewart, 1941, and Beattie, 1936) and methionine (McCarthy & Sullivan, 1941). In each case the substance is present practically entirely in chemical combination, choline as phospholipin, methionine in proteins.

In twenty unincubated eggs the total choline has been found to average 183 mg./egg (S.D. ± 22 mg.), and in eight unincubated eggs the total methionine has averaged 165 mg./egg (S.D. ± 11 mg.). Table 1 gives the results of the embryo analyses. Both compounds increase with incubation in the expected more or less smooth exponential manner, and methionine accumulates more rapidly than choline.

The analyses of the 'rests' show a progressive decrease, which in the case of methionine corresponds broadly to the increase in the embryo. In fact, up to the 18th day, the sum of the embryo and 'rest' methionine approximates closely to the total

methionine of unincubated eggs. At the end of incubation there is a fall, however, of about 25% in the total methionine of the egg, although the exponential increase in the embryo remains uninterrupted.

Table 1. *Analyses of embryos*

Incubation period (days)	Choline (mg./embryo)	Methionine (mg./embryo)
6	1	1
10	2	2·5
12	3	8
14	8	20
16	16	36
18	30	45
20	40	75

On the other hand, the total choline of the embryo and 'rest' is less than the choline of unincubated eggs as early as the 13th day, and by the 21st day the egg contains not more than 50% of the original 180 mg. choline. This decrease may be partly a coincidental reflection of the parallel change in lipoid phosphorus (Plimmer & Scott, 1908; Masai & Fukutomi, 1923; confirmed in this laboratory), but it appears probable also that choline is the only donor of methyl groups in the egg at least until the last 2 days of incubation. Such quantitatively important compounds as creatine and creatinine do not, however, account for more than 25% of the choline which disappears, even when the assumption is made that only one of the three choline methyl groups is available for transmethylation (cf. du Vigneaud, Chandler, Simmonds, Moyer & Cohn, 1946).

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The Behaviour of β-Glucuronidase and Nucleic Acids in Rat Liver during Growth. By G. T. Mills, Evelyn E. B. Smith, Beatrice Stary and I. Leslie. (*Biochemistry Department, University of Glasgow*)

A study has been made of the β-glucuronidase concentration in the livers of growing rats from before birth to maturity and in the livers of rats following subtotal hepatectomy.

It has been found that the liver β-glucuronidase concentrations are low in foetal rats, and that after birth there is a rise to a maximum around 20–40 days followed by a slight decline to an adult level above that for animals at birth. An examination of our data by the allometric method, which was first employed by Huxley (1924), indicates that the accumulation rate of β-glucuronidase is greater than that for liver tissue during the growth of the animal. However, the presence of other variables obscures the picture when liver weight is used as a base line and a much more precise index of reference than this is required.

Davidson & Leslie (1950) have demonstrated the value of the constancy of the deoxyribonucleic acid (DNA) content of the cell nucleus for a single species as an index of reference when studying growth phenomena. In the present work, by using the DNA content of the tissues as an indicator of cell number, along with the allometric method of analysis, the results indicate that the β-glucuronidase content per cell increases up to about 18 days postpartum and thereafter remains constant, and it is after this time that the growth rate of the liver is maximum. Our data reveal no relation between the rate of cell proliferation and the β-glucuronidase content per cell in the rat liver.

A study of rat liver regenerating after subtotal hepatectomy leads us to the same conclusion. In these experiments when the results are calculated on the basis of the DNA content of the tissue, a constant β-glucuronidase content per cell is found throughout the whole period of regeneration. During the 3 days following the operation when regeneration is most intense, the protein concentration of the liver is increasing rapidly while the β-glucuronidase concentration remains constant. It is only at about 6 days, when the protein concentration has returned to a constant level and regeneration is about 80% complete, that there is any significant rise in β-glucuronidase concentration.

The situation in the rat would therefore appear to be different from that in the mouse, where Levvy, Kerr & Campbell (1948) and Kerr, Campbell & Levvy (1949, 1950) have recorded a connexion between cell proliferation and the glucuronidase concentration of an organ. Further work is in progress to determine whether or not this is a true species difference.