REFERENCES

Armstrong, E. F. & Allan, J. (1924). *J. Soc. chem. Ind., Lond.*, **43**, 216*T*.
Brown, J. B. (1931). *J. biol. Chem.* **90**, 133.
Ellis, N. R. & Isbell, H. S. (1926). *J. biol. Chem.* **69**, 239.
Ellis, N. R., Rothwell, C. S. & Pool, W. O. (1931). *J. biol. Chem.* **92**, 385.
Ellis, N. R. & Zeller, J. H. (1930). *J. biol. Chem.* **89**, 185.
Gupta, S. S. & Hilditch, T. P. (1951). *Biochem. J.* **48**, 137.
Hilditch, T. P. (1947). *The Chemical Constitution of Natural Fats*, 2nd ed. London: Chapman and Hall.
Hilditch, T. P., Lea, C. H. & Pedelty, W. H. (1939). *Biochem. J.* **33**, 493.
Hilditch, T. P. & Maddison, L. (1948). *J. Soc. chem. Ind., Lond.*, **67**, 253.
Hilditch, T. P. & Pedelty, W. H. (1940). *Biochem. J.* **34**, 40.
Tveraaen, I. (1935). *Hvalrâd. Skr.* **11**, 5.

Observations on the Determination of the Specific Activity of the Inorganic Phosphate Fraction of Trichloroacetic Acid Extracts of Liver

BY A. H. ENNOR AND H. ROSENBERG
Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T.

(*Received* 22 *June* 1951)

The determination of the specific activity (S.A.) of inorganic phosphorus* present in a tissue extract involves its quantitative separation from organophosphates and the subsequent measurement of the radioactivity. Measurement of inorganic P is made directly on another sample. The method generally employed for separation involves a precipitation of the inorganic P either as a molybdenum salt, or as the ammonium magnesium salt, but the latter technique is generally preferred because of the high absorbing power of molybdenum for β-radiation (Hevesy, 1948). Because of the rather great sensitivity of the methods employed for the measurement of the radioactivity, careful attention to the conditions of precipitation is necessary. Prime among the requirements is that ample time should be allowed for completeness of precipitation and that sufficient carrier P be present. Generally the ammoniacal solution, from which inorganic P is being precipitated, is kept overnight at 0–5° to ensure complete precipitation.

There is some doubt about the amount of carrier P which should be added. The literature shows that most workers add carrier P, but it is rare to find any reference to the amount which has, in fact, been added. Theoretically, since it is a question of measuring only ^{32}P, the amount of non-radioactive P which is present is of no consequence, provided that sufficient has been added to ensure complete precipitation of ^{32}P, and that correction factors are applied should the precipitate being counted exceed a limiting thickness. From the practical viewpoint, however, the amount of carrier P which is present is of considerable importance and it is the purpose of this communication to show that serious errors are introduced into the determination of the S.A. by the use of conventional techniques. A preliminary report of this work has already been published (Ennor & Rosenberg, 1951). A method will also be described, by means of which the accurate and rapid determination of the S.A. of inorganic P may be made in presence of labile organophosphates.

A recent publication by Ennor & Stocken (1950) has given an account of a method for the determination of inorganic P in the presence of acid-molybdate-labile organophosphates. Briefly, this method, which is a modification of that described by Berenblum & Chain (1938), involves the combination of molybdic acid and inorganic P in an acid aqueous phase and the extraction of the resultant phosphomolybdic acid with *iso*butanol. Both reaction and extraction are accomplished in 10 sec. and thus only the P released in this time interval from any acid-molybdate-labile organophosphates present adds to the inorganic P. It was shown that the P so measured is subject to an error equal to only 1% of the amount of P present as phosphocreatine, this being the most labile P compound present in most tissue extracts. It occurred to us that this method would be of value in experiments involving ^{32}P, since the

* In this paper the term 'inorganic phosphate' refers to that form of phosphorus which can be precipitated as magnesium ammonium phosphate and which will react directly, in acid solution, with ammonium molybdate to form a reducible phosphomolybdate.

use of a liquid counter and a colorimeter would permit the determination of both the radioactivity and P on the one solution.

It should be pointed out that Rowles & Stocken (1950) have used a somewhat similar technique, except that the original method of Berenblum & Chain (1938) was followed. In general, however, the procedure described by Ennor & Stocken (1950) is to be preferred because of the presence of phosphocreatine in extracts of most tissues and in particular of liver (Barker, Ennor & Harcourt, 1951).

The subject-matter of this paper was presented at the Brisbane Meeting of the A.N.Z.A.A.S. in May 1951.

METHODS

Two adult male guinea pigs were used for the experiments. Both were starved for 24 hr. prior to sacrifice, and in the case of one animal 50μc. of ^{32}P was injected intraperitoneally 4 hr. before death. The ^{32}P was in the form of NaH_2PO_4 and was injected in a volume of 3·0 ml. to which sufficient non-radioactive NaH_2PO_4 had been added to render the solution isotonic. The other animal was untreated.

Preparation of liver extracts

The animals were stunned by a blow on the head and the cervical vessels severed. The abdomen was quickly opened and the liver removed and dropped into liquid air. The frozen liver was weighed and then crushed in a mortar previously chilled to the temperature of liquid air. It was then transferred to a Nalco-type homogenizer, roughly weighed and homogenized for 2 min. in 10% (w/v) trichloroacetic acid (TCA). The suspension was then centrifuged and the supernatant poured off. A further extraction was carried out on the residue and, after centrifugation, the supernatants were combined and filtered to remove small particles. Sufficient 5N-NaOH was then added to the extract to adjust the pH to 7·3. During and after these operations, the temperature of the extract was maintained at 0–5°. After neutralization the volume of the extracts was so adjusted that the equivalent of 1 g. of liver was contained in about 18 ml.

Precipitation and preparation of precipitates of magnesium ammonium phosphate

Samples (5·0 ml.) of all experimental solutions and TCA liver extracts investigated were carefully pipetted into 15 ml. tubes. Magnesia mixture (1·0 ml.) (Vogel, 1947) was added, together with carrier P in the form of NaH_2PO_4 in amounts as detailed later and then sufficient water to bring the volume to about 10 ml. 4N-NH_4OH was then added dropwise until the solution was alkaline to phenolphthalein. At this stage, 0·6 ml. of NH_4OH (sp.gr. 0·88) was added. Stirring was effected by means of an air stream from a fine capillary, care being taken to avoid scratching the walls, in order to obviate difficulty in quantitative removal of the precipitate later. The tubes were then placed in the refrigerator (1°) and left for 18–24 hr. At the end of this time the precipitates were quantitatively transferred to a circle (diam. = 2·5 cm.) of Whatman no. 50 filter paper, mounted in a stainless steel modified Büchner type funnel, as described by Oddie & Lynn (1951). The supernatant liquid was drawn through by suction and the precipitate was washed several times with 0·5N-NH_4OH and finally with ammoniacal ethanol. The filter pad was then removed and mounted on a brass disk and fixed in position by an annular brass ring. The precipitate was finally dried under an infra-red lamp before counting.

^{32}P and ^{31}P determinations

(1) *Magnesium ammonium phosphate precipitates.* ^{32}P was determined under standardized geometry using a conventional decade scaler, power pack and GM4 end-window counter. All samples were prepared and counted in quadruplicate and for such times as resulted in reduction of the statistical error to $\pm 1\%$.

For the determination of P two techniques were adopted, depending upon whether the precipitate was ashed or not. In those experiments demanding ashing, the entire filter-paper disk was carefully removed from the brass mount and transferred to a micro-Kjeldahl tube. 10N-H_2SO_4 (1·0 ml.) and conc. HNO_3 (1·0 ml.) were then added and the precipitate and paper wet-ashed on an open electric furnace. After all traces of HNO_3 were removed by continued heating, about 5 ml. of water were added and the tubes immersed in boiling water for 20 min. to hydrolyse any pyrophosphate formed. The contents were then quantitatively transferred to a measuring flask and made up to 50 ml. in the case of those precipitates containing 0·1 and 0·5 mg. of carrier. The precipitates containing 1·0 mg. of carrier P were made up to 100 ml. Duplicate 5·0 ml. samples were pipetted into 50 ml. separating funnels containing 2·0 ml. of 5% (w/v) NaCl. Other reagents were added in the volumes, concentrations, and by the technique described by Ennor & Stocken (1950). The higher concentrations described by these authors were used throughout and the 10 sec. shake technique employed. As it separated, the aqueous layer was run off into a 25 ml. measuring flask and made up to volume by the addition of water. The alcohol layer was then washed by vigorous shaking for 10 sec. with two successive volumes, each of 10 ml. of N-H_2SO_4. Each wash (wash 1 and wash 2) was drawn off as in the case of the aqueous layer and made up to a similar volume. Reduction was carried out by shaking for 30 sec. with 15 ml. of $SnCl_2$ (Berenblum & Chain, 1938) in flasks reserved for that purpose and the $SnCl_2$ layer, as before, was made up to 25 ml. by the addition of water. The alcohol phase was quantitatively washed into a 25 ml. flask with ethanol and made to volume with this solvent. Optical density of the alcohol phase was determined as described by Ennor & Stocken (1950). Radioactivity of the alcohol and all the aqueous phases was measured in a liquid counter of 10 ml. vol. and of the type described by Veall (1948).

In the case of those precipitates which were not ashed, the filter-paper disk was transferred to a volumetric flask of appropriate volume and the precipitate dissolved by the addition of 2 ml. of 0·1N-HCl. The solution was then adjusted to volume by the addition of water. Samples (5·0 ml.) were taken and treated as in the case of those precipitates which had been ashed.

(2) *TCA extract.* Usually for the determination of both ^{32}P and P in the inorganic P a 1·0 ml. sample was sufficient and the procedure followed was as described above for the ashed precipitates, except that the aqueous layer and the two washes were retained only in the case of the second model experiment. In the subsequent experiment the aqueous layer contained labelled organophosphates, and

these were of no interest in this experiment. After reduction, the alcohol phase was retained for optical density and radioactivity determinations.

Expression of results

Some of the experiments quoted here were carried out over 48–60 hr. and in such cases the observed counts have been corrected for decay to the time at which the first count was made. In all cases, appropriate corrections have been made to the counts as measured in *iso*butanol and water, and these corrected results have been expressed as what they would have been had they been counted as $MgNH_4PO_4$ using the end-window counter.

RESULTS

^{32}P *recoveries in model systems.* The initial experiment was designed to determine the amount of inactive sodium dihydrogen phosphate necessary to ensure complete precipitation of the ^{32}P from an aqueous solution as the magnesium ammonium salt. Three different amounts of carrier were added, namely, 0·1, 0·5 and 1·0 mg. of P as sodium dihydrogen phosphate. From Table 1 it may be seen that a tenfold increase in the amount of carrier has produced only about a 5% increase in the radioactivity of the precipitate, from which it may be inferred that precipitation is practically complete at the 1·0 mg. level. Additional data on the completeness of precipitation is given below.

Table 1. *The influence of varying amounts of inactive carrier* P *upon the amount of* ^{32}P *precipitated from an aqueous solution*

P added as inactive NaH_2PO_4 (mg.)	$MgNH_4PO_4$ ppt. counts/min.
0·1	930
0·5	950
1·0	980

In the second model experiment a TCA extract was prepared from the liver of a normal untreated guinea pig and radioactive phosphate was added after neutralization. This extract thus contained unlabelled organophosphates and inorganic P, some of which was labelled. The results (Table 2) indicate that, since no radioactivity could be detected in the aqueous layer from which the inorganic P had been extracted as phosphomolybdate, the *iso*butanol must quantitatively extract the ^{32}P. In addition, it is also evident that the partition coefficient for phosphomolybdate in *iso*butanol and water is so overwhelmingly in favour of the alcohol phase that no losses of radioactivity occur in the two acid washes and in the stannous chloride subsequent to extraction.

In the experiments carried out with magnesium ammonium phosphate precipitates prepared as a result of the addition of 0·1 and 1·0 mg. of P as carrier, a result similar to that shown in Table 1 is seen; i.e. a tenfold increase in the amount of carrier produces only a 3% increase in the radioactivity. When *iso*butanol extraction was carried out on the precipitates in the manner described above, the radioactivity was recovered in the alcohol phase and no counts in excess of background could be detected in the various aqueous phases. The

Table 2. *Recovery of* ^{32}P *from TCA extract of normal guinea pig liver*

(^{32}P was added to this extract after preparation. No labelled organophosphates were present. All counts are the means of duplicate determinations and in no case did the variation between duplicates exceed 3%.)

	Counts/min.	
*iso*Butanol extract of TCA	1070	
Aqueous layer, Wash 1, Wash 2, $SnCl_2$ layer	0	
	*a**	*b*†
$MgNH_4PO_4$ ppt.	990	1020
*iso*Butanol extract of ppt.	1030	1050
Aqueous layer, Wash 1, Wash 2, $SnCl_2$ layer	0	0

* In presence of 0·1 mg. P as NaH_2PO_4 as carrier.
† In presence of 1·0 mg. P as NaH_2PO_4 as carrier.

counts in the *iso*butanol and in the precipitates should, of course, be equal in both '*a*' and '*b*' respectively and the differences noted may be ascribed to experimental error. In view of the 3% increase in counts which is obtained when the amount of carrier P increases from 0·1 to 1·0 mg. it is of interest to note that even at the higher level the counts in the precipitate are still about 5% less than those recorded in the *iso*butanol extract of the TCA extract. Since this latter must be presumed to be correct (there is no radioactivity in the various aqueous phases) it may be inferred that the ^{32}P is only about 95% precipitated in the presence of 1·0 mg. of carrier P.

On the basis of the foregoing experiments it may be concluded that the *iso*butanol extraction of inorganic P as phosphomolybdate can be expected to give accurate results for the determination of the radioactivity and thus the specific activity of inorganic P both in the absence and presence of unlabelled organophosphates. When, however, the method was applied to TCA extracts of livers from animals injected with ^{32}P, the radioactivity, as measured in the *iso*butanol extract, was invariably less than that measured on the magnesium ammonium phosphate precipitates. In one case, the

counts/min./ml. of TCA extract, as measured on the precipitate, were almost double those determined on the *iso*butanol (Ennor & Rosenberg, 1951). On the basis of the foregoing experiments, the *iso*butanol extract cannot be expected to give a low result, but a high result is possible if it is assumed that some of the labelled organophosphates may go into simple solution in this solvent—this was considered unlikely. The position is very different in the case of magnesium ammonium phosphate, since here a low or a high result is possible by virtue of incomplete precipitation on the one hand and co-precipitation and/or adsorption on the other. The difference then between the magnesium ammonium phosphate count and the *iso*butanol count was regarded as significant and indicative of the presence of some radioactive contaminant.

In order to determine the source of error an experiment essentially similar in nature to the last described was carried out. In this case the TCA extract was prepared from the liver of an animal injected with ^{32}P and thus this extract contained, in addition to labelled inorganic P, labelled organophosphates.

Table 3. *Comparison of results obtained in determination of radioactivity (counts/min.) of inorganic P by precipitation as* $MgNH_4PO_4$ *and by extraction of phosphomolybdate in* isobutanol

(TCA extract prepared from liver of guinea pig injected intraperitoneally with approx. 50 μc. ^{32}P as NaH_2PO_4 3·5 hr. before death. All results are expressed as counts/min./ml. of TCA extract.)

*iso*Butanol extract of TCA		1130	
	*a**	*b**	*c**
$MgNH_4PO_4$ precipitate	1270	1600	1740
*iso*Butanol extract of precipitate	1140	1110	1100
Aqueous layer	150	430	570
Wash 1, wash 2, $SnCl_2$	0	0	0
*iso*Butanol extract of ashed precipitate	1240	1540	1680
Aqueous layer	0	0	0
Wash 1, wash 2, $SnCl_2$	0	0	0
*iso*Butanol extract of Mg precipitate + aqueous layer	1290	1540	1670

* To *a*, *b* and *c* was added respectively 0·1, 0·5 and 1·0 mg. of non-radioactive P as carrier and the counts are the means of quadruplicate determinations.

On the basis of the results described above, the figure 1130 (Table 3) may be taken as representing the true value for the radioactivity of the inorganic P, and it will be noted that this figure is exceeded if the radioactivity is determined on the magnesium ammonium phosphate precipitate. Of much greater significance, however, is the fact that the radioactivity in the precipitate increases as the amount of non-radioactive carrier increases. This increase in radioactivity can only be explained by the assumption that co-precipitation of some labelled organophosphate(s) has occurred. Proof of this assumption is given by determination of the radioactivity in the *iso*butanol extract of the precipitate. This technique, which was described above under the heading 'Magnesium ammonium phosphate precipitates' will extract only that P present as inorganic P, and any organophosphates present will remain in that fraction referred to as the 'aqueous layer'. Thus the radioactivity of the *iso*butanol extract of the magnesium ammonium phosphate should be equal to that of the TCA extract. The results indicate that this is the case (Table 3), and it will be noted that the counts are, within the limits of experimental error, identical for each of the three precipitates. On the other hand, the counts in the aqueous layer increase as the amount of carrier P increases and the sum of such counts, plus those in the corresponding *iso*butanol extract, are approximately equal to the counts in the respective precipitates.

Ashing of the precipitates and subsequent treatment of a sample with molybdate and extraction with *iso*butanol should yield an *iso*butanol extract which contains the same number of counts as did the original precipitate when counted by the end-window counter. In addition, since all the P in the precipitate is now in the form of inorganic P, no radioactivity should appear in the 'aqueous layer'. Both contentions are supported by the results and it must, therefore, be concluded that the magnesium ammonium phosphate precipitates are contaminated by some ^{32}P-containing organophosphorus compound(s).

It has been noted by Lehninger (1949) that some co-precipitation of adenosinetriphosphate (ATP) occurs when inorganic P is precipitated as magnesium ammonium phosphate and it therefore seemed likely that some, at least, of the excess counts in the precipitate would be due to ATP. For the purposes of comparison, the absorption spectra of one of each of the three precipitates prepared as a result of the addition of 0·1, 0·5 and 1·0 mg. of carrier P were determined by means of the Hilger 'Uvispek' spectrophotometer. The absorption curves were identical with that of an authentic sample of ATP and all had an absorption maximum at 2570 A. In our experiments no attempt has been made to identify positively the contaminating compound(s) as ATP by specific enzymic analysis, and it has been assumed that the entire absorption at 2570 A. is due to ATP and the results are expressed as such in Table 4. It is appreciated, however, that the precipitate may contain, in addition to ATP, both the mono- and di-phosphates.

It will be noted that the amount of 'ATP' which is found in the precipitates increases as the mass of the precipitate increases and that with the addition

of 1·0 mg. of P as inert carrier the amount precipitated reaches 0·3 mg. It is of interest to compare this with the figure of 0·79 mg. which was the amount of inorganic P found to be present in 5·0 ml. of the TCA liver extract.

Table 4. *Relationship between the amount of carrier P added and 'ATP' precipitated*

(The extinction coefficient at 2570 A. has been assumed to indicate only the presence of ATP and the results are expressed accordingly. All results are expressed on the basis of a 5·0 ml. sample of the TCA extract of guinea pig liver and the precipitates referred to are those whose radioactivities are indicated in Table 3.)

	*a**	*b**	*c**
Weight of $MgNH_4PO_4$ ppt. (mg.)	1·25	5·2	10·6
Weight of ATP (mg.)	0·056	0·206	0·300
Excess counts/min.†	700	2350	3050
Excess counts/min. / μg. ATP	12·5	11·4	10·2

* As in Table 3 columns *a*, *b* and *c* refer respectively to the precipitates containing 0·1, 0·5 and 1·0 mg. of carrier P.

† Excess counts/min. = counts/min. in $MgNH_4PO_4$ ppt. − counts/min. in *iso*butanol extract of the TCA liver extract.

Calculation of the contribution which is made to the total radioactivity of the precipitate by this 'ATP' shows a close parallelism to the weight of 'ATP'. The ratio, excess counts/min. to μg. 'ATP' indicates this more clearly and it is evident that, if only one ^{32}P-containing compound was co-precipitated and if this was 'ATP', this ratio should be a constant. The ratio is, however, not a constant and it may thus be inferred that all three of the phosphorylated adenosine compounds are precipitated to varying extents as the amount of carrier P increases, or that possibly some other ^{32}P-containing compound(s) is also precipitated. This inference gains support from the fact that the ratio, excess counts/min. to μg. 'ATP' (both are calculated on the difference which results when the amount of carrier P increases from 0·5 to 1·0 mg., i.e. *c*–*b*) falls to 7·4.

Table 5. *Specific activity* (S.A.) *of inorganic phosphate*

$$\text{S.A.} = \frac{\text{counts/min. TCA extract}}{\mu\text{g. P/ml. TCA extract}}.$$ Inorganic P = 15·9 μg./ml.

Method of determination of radioactivity		Counts/min. TCA extract	S.A.
On *iso*butanol extract of TCA extract		1130	71
On the $MgNH_4PO_4$ precipitate	+0·1 mg. carrier P	1270	81
	+0·5 mg. carrier P	1600	100
	+1·0 mg. carrier P	1740	110

Finally, it is of interest to calculate the specific activities of the inorganic P fraction of the TCA liver extract on the basis of the observed content of inorganic P = 15·9 μg.P/ml.

The S.A. which is regarded as the true value is that determined by means of the *iso*butanol-extraction technique. As indicated from the foregoing experiments, the value obtained for the S.A. as determined on the magnesium ammonium phosphate precipitates by solid-counting technique depends on the amount of non-radioactive carrier P which is added and in the case of the largest amount of carrier P which we have employed (1·0 mg.) the positive error introduced amounts to over 50 %.

DISCUSSION

In view of the fact that precipitation of inorganic P as magnesium ammonium phosphate is generally accepted as the most desirable method for the determination of the radioactivity and hence the specific activity of the inorganic P of tissue extracts, it was expected that some investigation would have been made of the specificity of the precipitation. To the best of our knowledge no such investigation has been carried out and indeed, apart from two occasions, no doubts appear to have been cast on the accuracy of the S.A. so determined. Kalckar, Dehlinger & Mehler (1944) qualified the interpretations of their data with the remark 'provided that the ammonium magnesium precipitate represents only inorganic P...'. Kamen & Spiegelman (1948), in discussing techniques for differentiating between 'ortho' and 'organic' P fractions, pointed out that the extent to which magnesia mixture will precipitate only 'ortho' P has still to be assessed. The experiments described in this communication show conclusively that the precipitate represents considerably more than inorganic P and that as a result the calculated S.A. is subject to considerable error. Since, however, the magnitude of this error is a function of the amount of inert carrier P which has been added, it is clearly impossible to assess the error which has been introduced into the results of any particular worker who has worked with ^{32}P, unless the amount of carrier P added is known. It would also seem certain that the true turnover rate of any particular organophosphorus compound must be greater than that quoted. This follows from the fact that the true S.A. of the inorganic P is less than that actually measured by conventional methods and that the S.A. of any particular organophosphate must, therefore, be a larger fraction of the true S.A. of the inorganic P than of the S.A. measured. The extent to which this argument is valid will depend on the compound, the methods used for its separation and on the composition of the extract. Thus, in the case of phosphocreatine, if the ^{32}P is freed by mild hydrolysis and then precipitated as the magnesium ammonium salt, the error introduced would conceivably be 'matched' to some extent by an error of similar magnitude in the case

of inorganic P. It is assumed, of course, that both hydrolyses and precipitations are carried out in unfractionated tissue extracts, but the argument may still hold if carried out on partially fractionated extracts. In this paper no attempt has been made to assess the error introduced with amounts of carrier in excess of 1·0 mg. P and our results indicate that precipitation of ^{32}P is incomplete at this level. Since other workers have claimed complete precipitation it may be assumed that the amount of carrier P added is greater than 1·0 mg. and hence that the results so obtained suffer from an error greater than 50%. In view of the doubts which arise as a result of the present work it would seem worthwhile to reinvestigate the turnover rates of those phosphorus-containing compounds whose precursors are believed to be inorganic P.

When this work was completed the work of Sacks (1951) came to our notice. He has stated that adenosine triphosphate-adenosinediphosphate is adsorbed on the magnesium precipitate and that it amounts to 20% of the total amount present. This statement, in the light of the present results, is ambiguous, since the amount of 'ATP' precipitated depends upon the bulk of the precipitate. The amount of ATP precipitated (as can be calculated from our results) can approach 100% of that present. In this connexion it is interesting to recall that Sacks (1940) was unable to precipitate ATP from an alkaline filtrate after precipitation of the inorganic P as magnesium ammonium phosphate. This failure may be explained by the present results and would seem due to the fact that all of the ATP was in the precipitate and not in the filtrate.

The procedure adopted by Sacks (1951) in dissolving the magnesium ammonium phosphate in acid, and precipitating the P as a molybdate, may also be expected to give erroneous results by virtue of the hydrolysis of the adenosinetriphosphate-adenosinediphosphate when heated in 2N-nitric acid at 60°.

The *iso*butanol technique detailed in the present paper is free from all the errors which characterize the conventional precipitation techniques, and has the added advantage that it is rapid and permits the determination of the radioactivity and the amount of P present in the one solution. The method has the additional advantage that it can be used for the determination of both P and ^{32}P released from any organophosphate by enzymic or chemical hydrolysis.

Addendum. A recent publication by Ernster, Zetterström & Lindberg (1950) has been brought to our notice. These workers have described a method for the determination of the specific activity of inorganic phosphate in tissue extracts which in principle is the same as that described here. Certain disadvantages are, however, inherent in the Martin & Doty (1949) modification of the Berenblum & Chain (1938) technique which was used. Thus the aqueous phase is in contact with the *iso*butanol-benzene extractant for 1 min. during which time hydrolysis of labile phosphorus compounds will occur. Also the practice of adding $SnCl_2$ in the presence of ethanol to form a single phase system with the *iso*butanol-benzene is not to be recommended, because of the extensive etching of glass cells and liquid counters which occurs. Ernster *et al.* (1950) have shown that their method, like ours, gives a lower result for the specific activity of inorganic phosphate than does the Mg precipitation technique. On the other hand, it does not seem to have been appreciated that the error is, in part at least, due to the presence of adenosine polyphosphates and that the magnitude of the error depends upon the amount of carrier P which has been added.

SUMMARY

1. The accepted technique of precipitating the inorganic phosphorus present in tissue extracts as magnesium ammonium phosphate for the determination of the radioactivity has been investigated and shown to be subject to considerable error.

2. The magnitude of this error depends on the bulk of the precipitate and is due to co-precipitation and/or adsorption of organophosphates among which are the adenosine polyphosphates.

3. The errors involved in determining the specific activity have been discussed, and it is concluded that the turnover rates of organophosphates as previously determined are probably lower than the true values.

4. A method has been described which permits the accurate and rapid determination of both the radioactivity and the amount of phosphorus present in the one solution.

It is a pleasure to express our thanks to Dr D. M. P. Phillips of this Department for determination of the absorption spectra and to Miss J. D'Alton for technical assistance. Our thanks are also due to Dr T. H. Oddie of the Radio Tracer Section of C.S.I.R.O. for advice on the preparation of samples of $MgNH_4PO_4$ for determination of radioactivity and for much assistance in the installation of the counting apparatus. It is also a pleasure to express our thanks to Dr F. G. Morgan, Director of the Commonwealth Serum Laboratories and to the Director General of Health, for a generous provision of laboratory space pending the establishment of this Department in Canberra.

REFERENCES

Barker, H. Ennor, A. H. & Harcourt, K. (1951). *Biochim. Biophys. Acta* (in the Press).
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Ennor, A. H. & Stocken, L. A. (1950). *Aust. J. Exp. Biol. Med. Sci.* **28**, 647.
Ennor, A. H. & Rosenberg, H. (1951). *Nature, Lond.*, **168**, 199.
Ernster, L., Zetterström, R. & Lindberg, O. (1950). *Acta chem. scand.* **4**, 942.
Hevesy, G. (1948). *Radioactive Indicators.* New York: Interscience.
Kalckar, H. M., Dehlinger, J. & Mehler, A. (1944). *J. biol. Chem.* **154**, 275.
Kamen, M. D. & Spiegelman, S. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 151.
Lehninger, A. L. (1949). *J. biol. Chem.* **178**, 625.
Martin, J. M. & Doty, D. M. (1949). *Anal. Chem.* **21**, 965.
Oddie, T. H. & Lynn, A. R. (1951). *C.S.I.R.O. Tracer Element Report No.* 1. *Preparation of Filtered Samples for Radioactive Assay.*
Rowles, S. L. & Stocken, L. A. (1950). *Biochem. J.* **47**, 489.
Sacks, J. (1940). *Amer. J. Physiol.* **129**, 227.
Sacks, J. (1951). *Arch. Biochem.* **30**, 423.
Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.
Vogel, A. (1947). *Quantitative Inorganic Analysis.* London: Longmans.

Some Effects of Administering Ergothioneine to Rats

By H. HEATH, C. RIMINGTON, C. E. SEARLE
University College Hospital Medical School, London, W.C. 1

and A. LAWSON
Royal Free Hospital School of Medicine, London, W.C. 1

(*Received* 27 *July* 1951)

The presence of ergothioneine in erythrocytes is well established. This has been made possible by the delicate and highly specific Hunter diazo reaction (Hunter, 1928, 1949). Latner (1948) used a modified diazo reaction to determine ergothioneine in blood ultrafiltrates, while Lawson, Morley & Woolf (1950) precipitated ergothioneine with iodobismuthous acid prior to colorimetric determination by the Hunter reaction. Touster (1951) has published a method for the estimation of ergothioneine in which the thiol group is oxidized with bromine water to sulphate, which is precipitated as benzidine sulphate and determined colorimetrically. The reaction of certain mercaptoglyoxalines with 2:6-dichloroquinone-chloroimide described by McAllister (1951*a*, *b*) might also be applicable to the determination of ergothioneine, but like Touster's method would not have the specificity of the Hunter reaction.

Ergothioneine has been stated to occur in human, rat, pig, ox, cat, guinea pig, dog, fowl and pigeon erythrocytes in amounts up to 30 mg./100 ml. of whole blood. There is no report in the literature of any species in which ergothioneine is entirely absent. It is found exclusively in the red cells and is not present in the plasma (cf. Fraser, 1950). Variations of ergothioneine blood levels with geographical location have been recorded by Hunter (1951) for men, rats and pigs. Significant increases in diabetic patients have been reported by Salt (1931) and by Fraser (1950). Latner & Mowbray (1948) observed lower ergothioneine levels in some thyrotoxic patients and raised levels in some cases of myxoedema.

Ergothioneine is the only 2-mercaptoglyoxaline known to occur naturally, and has been found only in blood, in ergot and in some other fungi. See, however, Leone & Mann, 1951. The possibility that erythrocyte ergothioneine is derived from dietary ergothioneine seems to be remote, and the report of Eagles & Vars (1928) that a maize diet increased the blood ergothioneine level in pigs has not been confirmed by Hunter (1951). Hunter also showed that when ergothioneine or 2-thiolhistidine was given to young rats (5 mg./animal) for 3 days, there was no increase in blood ergothioneine 2 days later. As will be shown later, however, the erythrocyte concentration of ergothioneine can be greatly increased by feeding rats a diet containing 0·1 % ergothioneine, although the increase is gradual.

The question of the occurrence of ergothioneine in urine has been examined by various workers. Sullivan & Hess (1933) and Work (1949) claimed that it was excreted in appreciable amounts, whilst Woolf (1949) and Lawson *et al.* (1950) failed to detect any by paper chromatography. The last named workers also reported that a single injection of ergothioneine in the rat did not bring about any detectable excretion during the following 24 hr. The use of a new procedure which has likewise given no indication of the presence of ergothioneine in