green filter with maximum transmittance in the range 540–600 m μ . The amount of N in each sample was obtained by comparison with a standard. The corresponding protein concentration was calculated, taking the N content obtained to be 16.5% of the dry solid feather keratin originally dissolved (Woodin, 1954).

RESULTS AND DISCUSSION

The specific rotation, $[\alpha]_D^{25}$, of soluble feather keratin was -430° at 25° . The estimated probable error is $\pm 45^\circ$. This was for a solution of protein concentration 3 mg./ml. The result of these experiments therefore clearly shows that the solution of soluble feather keratin has a large negative specific rotation that is of the same order of magnitude as that of collagen. The specific rotation of collagen (ichthyocol) in citrate buffer at 10° , $[\alpha]_D^{10}$, has been found by Cohen (1955) to be $-350^\circ \pm 30^\circ$. In general both collagen and the triple helical polypeptides poly-L-proline and poly-L-hydroxyproline exhibit a large negative specific rotation, $[\alpha]_D$, being in the range of -300° to -400° (Sasisekharan, 1961). On the other hand polypeptides of the α -helical form generally have a specific rotation, $[\alpha]_D$, in the range 0 – 50° , whereas for wool (α -keratin) $[\alpha]_D$ is of the order of -50° to 0° . In general, α -type proteins have a small negative rotation, whereas in the random-coiled or denatured form they have a specific rotation of the

order of -100° . The fact that feather keratin exhibits a large negative rotation of -430° provides further evidence that it has a structure similar to that of collagen. The optical-activity data also support the triple helical structure of feather keratin.

SUMMARY

1. The optical rotation of soluble feather keratin has been studied, and the specific rotation of the protein in solution at 25° , $[\alpha]_D^{25}$, has been found to be -430° . This value is very close to that observed for soluble collagen and the triple helical polypeptides, and very different from that observed for α -proteins. It thus supports the triple helical structure put forward for feather keratin from X-ray data by Ramachandran & Dweltz (1961).

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Insulin and Incorporation of Amino Acids into Protein of Muscle

CELLULAR AMINO ACID LEVELS AND AMINOISOBUTYRIC ACID UPTAKE

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Kipnis & Noall (1958) found that insulin *in vitro* enhances the accumulation of a non-utilizable amino acid, α -aminoisobutyric acid, by isolated rat diaphragm. This observation has led to the belief that insulin may bring about the stimulation of incorporation of amino acids into protein of diaphragm (Sinex, MacMullen & Hastings, 1952; Krahle, 1953; Manchester & Young, 1958a; Wool & Krahle, 1959a) by a promotion of entry of amino acids into the tissue. However, in opposition to this view, no significant effect of insulin on the accumulation of naturally occurring amino acids has been observed and there is substantial evidence

that insulin can stimulate incorporation of amino acids into protein under conditions in which it is not affecting their transfer into the cell (Wool & Krahle, 1959b; Manchester & Krahle, 1959; Manchester & Young, 1960). The possibility has been put forward (Manchester, 1960, 1961; Tepperman & Tepperman, 1960) that the enhanced uptake of α -aminoisobutyric acid in the presence of insulin arises as a consequence of an increased rate of protein synthesis and that the α -aminoisobutyric acid enters the cell to take the place of the extra amino acids incorporated into protein. This possibility rests on the tacit assumption that an in-

creased rate of incorporation of labelled amino acids into protein does indeed represent an increased rate of protein synthesis. But, on the evidence at present available, an increased incorporation of labelled amino acids into protein of diaphragm could equally arise if insulin were merely to facilitate the access of amino acids from the medium to the site of protein synthesis, without changing the total amount of protein formed.

The purpose of this paper is to provide evidence to the effect that in stimulating amino acid incorporation insulin does increase the amount of protein synthesized and to discuss experiments designed to investigate the mechanism by which insulin enhances the uptake of aminoisobutyric acid.

METHODS

Materials. Diaphragm was taken from female albino Wistar rats, 100–120 g., fed on a stock laboratory diet (Bruce & Parkes, 1949) and allowed free access to food and water at all times. The hemidiaphragms were incubated in Krebs bicarbonate buffer (Krebs & Henseleit, 1932), gassed with $O_2 + CO_2$ (95:5), pH 7.4 at 37°. The buffer contained no glucose or other additions unless otherwise stated.

The insulin used (Boots Pure Drug Co. Ltd.) was a six-fold recrystallized sample of potency of about 20 units/mg. It was dissolved in $N/300$ -HCl to a concentration of 20 units/ml. and diluted with buffer as required. Alkali-inactivated insulin was prepared as described by Sutherland & Cori (1948). Insulin (1 mg.) was heated at 37° for 2.5 hr. in 1 ml. of 0.08N-KOH. It was subsequently diluted with buffer to the required concentration. The effectiveness of the inactivation was judged by the inability of the sample to stimulate either amino acid incorporation or glucose uptake by isolated diaphragm. The serum albumin was a highly purified specimen from rat plasma, kindly provided by Dr P. N. Campbell (Courtauld Institute of Biochemistry, Middlesex Hospital, London). It had been separated from α_1 -globulin by electrophoresis on cellulose acetate. Casein was a commercial preparation (British Drug Houses Ltd.). Tolbutamide (*N*-butyl-*N'*-toluene-*p*-sulphonylurea) was from Burroughs Wellcome and Co.; 1:2-dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzene was kindly provided by Professor E. F. Gale (Department of Biochemistry, University of Cambridge). Proflavin (as the hemisulphate) was from British Drug Houses Ltd. α -Aminoiso[1- ^{14}C]butyric acid, [1- ^{14}C]glycine and [8- ^{14}C]adenine were obtained from The Radiochemical Centre, Amersham, Bucks.

Measurement of release of amino acids. In two beakers each containing 20 ml. of buffer at 0° were collected hemidiaphragms from six rats, each beaker containing a hemidiaphragm from each rat. After standing for a few minutes at 0° the six hemidiaphragms from each beaker were blotted and transferred to a 50 ml. conical flask in which was 6 ml. of buffer containing the additions stated in Tables 1–3. The second flask in each experiment acted as the control for the first. The flasks were quickly gassed with $O_2 + CO_2$, sealed and incubated at 37° with shaking in a water bath. Samples (0.2 ml.) of medium were taken from each flask at 15, 30, 60 and 90 min., the flasks being quickly

gassed after the taking of each sample. At 120 min. a final sample of medium was taken, the six hemidiaphragms from each flask were weighed (about 600 mg.) and placed in 10 ml. of water in a boiling tube. Each tube was placed in a boiling-water bath for 5 min., with occasional shaking, then cooled and a sample (0.2 ml.) taken. The samples of medium and tissue extract, as well as samples of buffer in which the diaphragms were soaked before incubation, a blank and a sample of a solution of valine containing 21 μ g. of amino nitrogen/ml., were deproteinized by the addition of 3.5 ml. of $N/70$ - H_2SO_4 followed by 0.3 ml. of 0.67% sodium tungstate soln. After the solutions had stood for 1 hr. any slight precipitate was removed by centrifuging, and a sample (2 ml.) from each taken for the amino acid estimation. For the results in Table 4 single hemidiaphragms were incubated for 2 hr., each in 1 ml. of medium. After incubation each hemidiaphragm was weighed and heated for 5 min. in a boiling-water bath in 2 ml. of water. Samples (0.2 ml.) of extract and medium were taken and deproteinized as described above.

The method for the estimation of amino acids was essentially that of Chibnall, Mangan & Rees (1958). To each sample was added 1 ml. of a buffer made up of 23 ml. of 5M-sodium acetate (pH 5.5), 2 ml. of 0.01M-KCN and 75 ml. of 2-methoxyethanol (methyl Cellosolve; Hopkin and Williams Ltd.). Ninhydrin (0.2 ml. of 10%, w/v, solution in 2-methoxyethanol) was then added and the samples were heated in a boiling-water bath for 20 min. They were then cooled, diluted to 10 ml. with 60% ethanol and the light-absorption at 570 $m\mu$ was measured in a spectrophotometer. Experience showed that only small quantities of amino acids were liberated during the initial soaking. These quantities have been left out of all calculations.

Measurement of the accumulation of aminoisobutyric acid. Measurement of the accumulation of α -aminoiso[^{14}C]butyric acid was carried out as described by Manchester & Young (1960). Hemidiaphragms (four per flask, in which was 4 ml. of buffer containing aminoisobutyric acid at a concentration of 0.22 μ mole/ml. and specific activity of about 1.8 μ C/ μ mole, except where otherwise stated: Table 7) were incubated for 1.5 hr. at 37°. At the end of this period each hemidiaphragm was blotted, weighed and placed separately in 2 ml. of water. The tubes containing hemidiaphragms were heated for 5 min. in a boiling-water bath and then cooled. A sample (0.5 ml.) of medium was diluted to 5 ml. with water. Samples (1 ml.) of tissue extract and diluted medium were pipetted on to 2 cm. diam. aluminium disks, previously painted with a metal lacquer, and the liquid was removed by evaporation under an infrared lamp. The content of radioactivity of the disks was assessed in a Panex counter (Model D-554). Counts ranged between 500 and 3000/min.

Measurement of incorporation of [^{14}C]glycine into protein. The method for the measurement of incorporation of [^{14}C]glycine into protein was very similar to that described by Manchester & Young (1958a). Glycine was added to the medium at a concentration of 1 μ mole/ml. (specific activity about 80 μ C/m-mole) unless otherwise stated (Table 8). After incubation for 2 hr. each hemidiaphragm was first heated for 5 min. and then homogenized in about 10 ml. of 10% trichloroacetic acid. The precipitate was spun down and resuspended in about 10 ml. of 10% trichloroacetic acid and heated for 15 min. at 90°. The insoluble material was

collected and dissolved in 1.5 ml. of 0.4N-NaOH and reprecipitated by addition of 10% trichloroacetic acid. It was washed three times with acetone and finally suspended in a small quantity of ether and poured on to a tared aluminium disk, area about 7.5 cm.² The disks were allowed to dry, then weighed, and the content of radioactivity was assessed in a Nuclear-Chicago gas-flow counter (model C-110B). Each protein sample weighed about 15 mg. Since the sample weights were fairly constant no corrections for self-absorption were made in calculating specific activities.

Measurement of incorporation of [¹⁴C]adenine into nucleic acid. [¹⁴C]Adenine was added to the medium at a concentration of 0.01 μ mole/ml. (specific activity about 5 μ C/ μ mole). After incubation for 2 hr. each hemidiaphragm was homogenized in about 10 ml. of cold 10% trichloroacetic acid containing 0.1% of [¹⁴C]adenine. The insoluble material was washed three times with cold 10% trichloroacetic acid containing [¹⁴C]adenine, three times with acetone and poured in ether on to tared disks as described above.

Measurement of release of potassium. Release of potassium was determined by flame photometry as described by Randle & Smith (1958).

Measurement of uptake of glucose. Uptake of glucose was calculated from the disappearance of glucose from the medium. After incubation the diaphragm was blotted and weighed and the residual glucose in the medium determined colorimetrically by Somogyi's modification of Nelson's method (Somogyi, 1945).

RESULTS

Effect of insulin on amino acid concentrations in diaphragm

Kline (1949) found that during incubation of isolated rat diaphragm there was a release of amino acids into the medium. If insulin stimulates incorporation of amino acids by increasing the rate of protein synthesis, then in the presence of the hormone the rate of accumulation of amino acids in the medium should be decreased, either because the rate of exit declines or because a greater uptake of amino acids is required to sustain the increased rate of protein synthesis.

Release of free amino acids by isolated rat diaphragm during incubation was found to follow the pattern described by Kline (1949), about 40% of the amino acids released during the incubation period of 2 hr. appearing in the medium in the first 15 min. (Table 1). Addition of insulin to the medium produced a small decrease in the amount of amino acids released and, moreover, the absolute size of the insulin effect increased with time (Table 1). Addition of alkali-inactivated insulin, highly purified serum albumin, casein or glucose did not have any consistent effect on the release of amino acids by isolated diaphragm (Table 1).

Table 1. *Effect of insulin, various proteins and glucose on the release of amino acids by isolated rat diaphragm during incubation*

For details see Methods.					Amino acid released (μ g. of amino nitrogen/g. of tissue incubated)				
Incubation time (min.)	...	...	...	...	15	30	60	90	120
Additions to the medium									
1.* No addition					146	186	251	302	355
Insulin [5 μ g. (0.1 unit)/ml.]					139	172	222	268	310
Decrease produced by insulin (Range)					7 (3-11)	14 (10-20)	29 (15-40)	34 (29-44)	45 (29-57)
2. No addition					132	183	246	310	363
Inactivated insulin (5 μ g./ml.)					136	183	259	307	358
Decrease produced by inactivated insulin (Range)					-4 (-9-0)	0 (-14-18)	-13 (-25-7)	3 (-27-23)	5 (-20-21)
3. No addition					155	186	259	316	365
Serum albumin (100 μ g./ml.)					156	186	257	298	357
Decrease produced by albumin (Range)					-1 (-10-7)	0 (-5-5)	2 (-1-5)	18 (10-26)	8 (1-15)
4. No addition					167	224	290	357	418
Casein (250 μ g./ml.)					156	210	275	349	410
Decrease produced by casein (Range)					11 (5-16)	14 (-3-26)	15 (5-25)	8 (-11-27)	8 (-24-39)
5. No addition					167	223	290	341	390
Glucose (2.5 mg./ml.)					170	229	286	354	387
Decrease produced by glucose (Range)					-3 (-4 to -2)	-6 (-17-5)	4 (-13-21)	-13 (-20 to -6)	3 (-36-42)

* 1 and 2 are each the mean of three experiments, each involving six pairs of hemidiaphragms; 3, 4 and 5 are each the mean of two experiments.

Although the diminution of release of amino acids brought about by insulin was small, in each experiment the difference was remarkably consistent and decisive by comparison with the figures obtained with the inactivated insulin, serum albumin, casein or glucose. A significant diminution in the release of amino acids after 2 hr. of incubation in the presence of insulin is shown in Table 4.

Since the release of amino acids during the first 15 min. of incubation may arise largely as the result of damage sustained by the tissue during excision, Kline (1949), who was using the release of amino acids from isolated diaphragm as a measure of the rate of protein catabolism, based his calculations

on the rate of release of amino acids after 15 min. It will be seen (Table 2) that, when from the results in Table 1 the rate of release of amino acids by diaphragm is calculated for periods after the first 15 min., a diminution in the presence of insulin is again apparent. As in Table 1, no consistent effect of addition of inactivated insulin, serum albumin, casein or glucose is observable in Table 2.

The suppression by insulin of the release of amino acids by diaphragm during incubation does not result from an increased retention of amino acids in the cell pool, for higher concentrations of amino acids in diaphragm were not observed in the

Table 2. *Effect of insulin, various proteins and glucose on the release of amino acids by isolated rat diaphragm during incubation after the first 15 min. of incubation*

Additional incubation time (min.)	Amino acids released (μ g. of amino nitrogen/g. of tissue incubated) after further incubation, less that released in the first 15 min. of incubation			
	15	45	75	105
Additions to the medium				
1. No addition	40	105	156	209
Insulin [5 μ g. (0.1 unit)/ml.]	33	83	129	171
Decrease produced by insulin	7	22	27	38
2. No addition	51	114	178	231
Inactivated insulin (5 μ g./ml.)	47	123	171	222
Decreased produced by inactivated insulin	4	-9	7	9
3. No addition	31	104	161	210
Serum albumin (100 μ g./ml.)	30	101	142	201
Decrease produced by albumin	1	3	19	9
4. No addition	57	122	190	251
Casein (250 μ g./ml.)	54	118	193	254
Decrease produced by casein	3	4	-3	-3
5. No addition	56	123	174	223
Glucose (2.5 mg./ml.)	59	116	184	217
Decrease produced by glucose	-3	7	-10	6

Table 3. *Effect of insulin, various proteins and glucose on the quantity of free amino acids found in isolated rat diaphragm and the incubation medium after incubation for 2 hr.*

Additions to the medium	Amino acids found (μ g. of amino nitrogen/g. of tissue incubated)			Decrease (%)
	In tissue	In medium	Total	
1.* No addition	430	355	785	11.7
Insulin [5 μ g. (0.1 unit)/ml.]	383	310	693	
2. No addition	271	363	634	-1.1
Inactivated insulin (5 μ g./ml.)	283	358	641	
3. No addition	371	365	736	-1.0
Serum albumin (100 μ g./ml.)	386	357	743	
4. No addition	272	418	690	0.5
Casein (250 μ g./ml.)	277	410	687	
5. No addition	390	371	761	1.2
Glucose (2.5 mg./ml.)	387	365	752	

* 1 and 2 are each the mean of three experiments, each involving six pairs of hemidiaphragms; 3, 4 and 5 are each the mean of two experiments.

presence of the hormone (Table 3). Rather, the amount of amino acids in the tissue was less when it was incubated for 2 hr. in the presence of insulin than when the insulin was omitted (Table 3). Addition of inactivated insulin, serum albumin, casein or glucose had no similar effect (Table 3). When the amount of amino acids in the medium and that remaining in the tissue after incubation for 2 hr. are added together it is found that the

quantity of amino acids in the system is significantly less in the presence of insulin than in its absence (Tables 3 and 4). No comparable changes were produced by inactivated insulin, serum albumin, casein or glucose (Table 3). Thus insulin can not only depress the accumulation of amino acids in the medium, but also diminishes the amount of amino acids in the system as a whole (those in the tissue and the medium added together).

Table 4. *Effect of insulin on the quantity of free amino acids found in isolated rat diaphragm and the incubation medium after incubation*

Similar to Table 3, no. 1, except that each hemidiaphragm was incubated separately for 2 hr. in 1 ml. of medium in order that sufficient observations might be obtained for statistical analysis. Each figure is the mean $\pm$ s.e. of the mean of eight observations. The value of *P* for a difference which is significant is indicated.

Addition to the medium	Amino acids found (μ g. of amino nitrogen/g. of tissue incubated)		
	In tissue	In medium	Total
No addition	429 $\pm$ 15	405 $\pm$ 15	834 $\pm$ 9
Insulin [5 μ g. (0.1 unit)/ml.]	422 $\pm$ 14	354 $\pm$ 16	776 $\pm$ 17
		<i>P</i> < 0.05	<i>P</i> < 0.01

Table 5. *Effect of insulin, various proteins and glucose on the ratio of concentration of amino acids within isolated diaphragm to that in the medium after incubation for 2 hr.*

Concn. of amino acids without additions				Concn. of amino acids with additions shown		
	In tissue (μ g. of amino nitrogen/g. of tissue water*)	In medium (μ g. of amino nitrogen/ml. of medium)	Ratio	In tissue (μ g. of amino nitrogen/g. of tissue water*)	In medium (μ g. of amino nitrogen/ml. of medium)	Ratio
With insulin [5 μ g. (0.1 unit)/ml.]						
Expt. 1	747	46.0	16.2	558	42.8	13.0
Expt. 2	509	46.3	11.0	504	37.3	13.5
Expt. 3	467	37.1	12.6	466	30.7	15.2
	Average		13.3	Average		13.9
With inactivated insulin (5 μ g./ml.)						
Expt. 1	313	40.4	7.8	367	36.5	10.0
Expt. 2	346	39.7	8.7	325	36.7	8.8
Expt. 3	425	44.6	9.5	440	43.6	10.1
	Average		8.7	Average		9.6
With serum albumin (100 μ g./ml.)						
Expt. 1	423	39.8	10.6	448	39.5	11.3
Expt. 2	320	36.2	8.8	325	37.4	8.7
	Average		9.7	Average		10.0
With casein (250 μ g./ml.)						
Expt. 1	357	39.4	9.1	380	38.4	10.1
Expt. 2	369	37.5	9.8	357	35.8	10.0
	Average		9.5	Average		10.0
With glucose (2.5 mg./ml.)						
Expt. 1	399	40.3	9.9	384	42.5	9.0
Expt. 2	343	42.4	8.1	347	40.9	8.5
	Average		9.0	Average		8.8

* Tissue water has been assumed to be 75% of wet weight.

Since the quantity of amino acids found in the tissue is not raised in the presence of insulin, the ratio of the concentration of amino acids in the tissue to that in the medium therefore remains unchanged. The ratios shown in Table 5, although they vary considerably from one experiment to another, do not provide any evidence to suggest that insulin promotes the accumulation of the naturally occurring amino acids and ninhydrin-reacting peptides of diaphragm.

The release of amino acids by isolated diaphragm is generally thought of as a measure of the extent to which protein breakdown exceeds protein synthesis in this preparation. However, as the figures in Table 6 indicate, the major part of the amino acids appearing in the medium during incubation represents the diminution of the pool of free amino acids in the tissue, the cell pool of free amino acids in isolated hemidiaphragm undergoing a considerable reduction in size. Thus 80% of the amino nitrogen content of the system at the end of the incubation was present as free amino nitrogen at the beginning (Table 6).

Effect of insulin on accumulation of aminoisobutyric acid

It has been suggested (Manchester, 1960, 1961; Tepperman & Tepperman, 1960) that the increased accumulation of α -aminoisobutyric acid by diaphragm observed in the presence of insulin arises, not from an action of insulin on the accumulation of amino acids, but as a consequence of the acceleration by insulin of the rate of protein synthesis. This suggestion assumes that amino acids can be treated as a class of compounds which for accumulation purposes are capable of replacing and displacing each other. The work of Christensen and his colleagues (summarized in Christensen, 1960) supports this assumption. Because of the increased rate of utilization of amino acids from the cell pool for protein synthesis in the presence of insulin, the rate of release of amino acids declines and the rate of entry or re-entry increases in order

to preserve a constant concentration gradient between the tissue and the medium. As α -aminoisobutyric acid is an amino acid it is subject to the same fluxes and movements as the other amino acids. Because aminoisobutyric acid is not incorporated into protein it accumulates in the tissue and, by acting as the replacement for the extra amino acids incorporated into protein in the presence of insulin, so it is progressively accumulated over a period of time under the influence of the hormone.

If this hypothesis is correct it should be open to experimental verification in at least two ways. First, if a naturally occurring amino acid is added to the medium in large quantity then this amino acid rather than the trace quantities of aminoisobutyric acid present should make up the deficiency caused, in the presence of insulin, by the amino acids entering protein, and under these conditions an effect of insulin on the accumulation of aminoisobutyric acid would not be expected. As a corollary of this, if the quantity of aminoisobutyric acid added to the medium is increased, then the quantity entering the tissue in the absence of insulin will rise and any extra amino acids incorporated into protein will become negligible in quantity by comparison with the basal uptake. Thus it is to be expected that, as the quantity of aminoisobutyric acid added to the medium is increased, the size of the insulin effect will diminish, and this is in fact observed (Table 7). Increasing the concentration of aminoisobutyric acid added to the medium from 0.2 to 20 μ moles/ml. progressively obscures an effect of insulin on its accumulation by diaphragm. By comparison, a significant stimulation of incorporation of [14 C]glycine into protein is observable at concentrations of glycine as great as 20 μ moles/ml. (Table 8).

In a study of the effect of the addition of other amino acids to the medium on the accumulation of aminoisobutyric acid it was found (Table 9) that addition of L-valine, L-leucine, glycine and L-alanine at 20 mM concentration decreased very

Table 6. *Release of amino acids by isolated rat diaphragm during incubation*

Number of observations is shown in parentheses. Tissue values are means $\pm$ s.e. Release of amino acids into the medium by 479 mg. of hemidiaphragm was $(21.5 \times 6) = 129 \mu$ g. of amino nitrogen = 270 μ g./g. of tissue.

Amino nitrogen content of tissue and medium before incubation = 452

Amino nitrogen content of tissue and medium after incubation = 282 + 270 = 552

Therefore increase in amino nitrogen content of the system during incubation = 100 μ g./g. of tissue.

	Intact diaphragms	Hemidiaphragms
Content of diaphragm before incubation (μ g. of amino nitrogen/g. of tissue) (6)	522 $\pm$ 9	452 $\pm$ 15
Content of diaphragm after incubation for 1.5 hr. (6)	455 $\pm$ 11	282 $\pm$ 9
Decrease	67	170
Content of medium after incubation (μ g. of amino nitrogen/ml. of medium)	54.5	21.5
Ratio of internal concn. to external concn.	8.3	13.1

substantially the size of the effect of insulin on the accumulation of α -aminoisobutyric acid. Leucine, glycine and alanine at 2 mM also had a considerable effect in this respect, although addition of valine at 2 mM concentration did not. On the other hand, even in the presence of 20 mM concentration of

amino acid there was a significant, even if smaller, stimulation by insulin of the accumulation of aminoisobutyric acid.

The second way in which experimental verification of the hypothesis advanced above has been sought is based on the argument that, under condi-

Table 7. *Effect of change of concentration of α -aminoiso[^{14}C]butyric acid on the stimulation by insulin of its accumulation by isolated rat diaphragm*

Each figure is the mean $\pm$ s.e. of the mean of four observations. Value of P for a difference between means which is significant is indicated.

Concn. of aminoisobutyric acid added to the medium (mM)	Ratio: $\frac{\text{Concn. of aminoisobutyric acid in tissue water}}{\text{Concn. of aminoisobutyric acid in incubation medium}}$			Significance of insulin effect (P)
	No added insulin	Insulin added (0.1 unit/ml.)	Difference	
0.22	2.04 ± 0.13	4.08 ± 0.11	2.04	< 0.001
2.0	1.45 ± 0.076	2.08 ± 0.12	0.63	< 0.01
20.0	1.08 ± 0.020	1.10 ± 0.024	0.02	—

Table 8. *Stimulation by insulin of the incorporation of [^{14}C]glycine at varying concentrations into protein of isolated rat diaphragm*

Each figure is the mean $\pm$ s.e. of the mean of six observations. The stimulation by insulin is in each case significant ($P < 0.01$). Quantity of radioactivity added to the medium was in each case the same ($0.08 \mu\text{C/ml.}$).

Concn. of glycine added to the medium (mM)	Radioactivity in diaphragm protein (counts/min./mg.)		Stimulation (%)
	No added insulin	Insulin added (0.1 unit/ml.)	
0.003	44.6 ± 2.22	82.2 ± 7.29	85
2	11.1 ± 0.66	16.1 ± 0.57	45
20	1.76 ± 0.10	2.62 ± 0.20	49

Table 9. *Effect of addition of other amino acids on the accumulation of α -aminoiso[^{14}C]butyric acid by isolated rat diaphragm and the influence of insulin on this process*

Each figure is the mean $\pm$ s.e. of the mean of four observations.

Additions to the medium	Ratio: $\frac{\text{Concn. of aminoisobutyric acid in tissue water}}{\text{Concn. of aminoisobutyric acid in incubation medium}}$			Significance of insulin effect (P)
	No added insulin	Insulin added (0.1 unit/ml.)	Difference	
1. No addition	(a) 2.65 ± 0.09	5.35 ± 0.29	2.70	< 0.001
L-Valine (2 mM)	(b) 2.56 ± 0.08	5.00 ± 0.20	2.44	< 0.001
L-Valine (20 mM)	(c) 1.10 ± 0.08	1.78 ± 0.10	0.68	< 0.01
Significance of differences	(a) - (c) $P < 0.001$	(a) - (c) $P < 0.001$		
2. No addition	(a) 2.24 ± 0.06	4.31 ± 0.12	2.07	< 0.001
L-Leucine (2 mM)	(b) 2.20 ± 0.11	3.37 ± 0.12	1.17	< 0.001
L-Leucine (20 mM)	(c) 1.63 ± 0.06	2.49 ± 0.20	0.86	< 0.01
Significance of differences	(a) - (c) $P < 0.001$	(a) - (b) $P < 0.01$ (a) - (c) $P < 0.001$		
3. No addition	(a) 1.92 ± 0.06	4.08 ± 0.32	2.16	< 0.001
Glycine (2 mM)	(b) 1.70 ± 0.11	2.58 ± 0.34	0.88	< 0.05
Glycine (20 mM)	(c) 1.21 ± 0.04	1.64 ± 0.06	0.43	< 0.001
Significance of differences	(a) - (c) $P < 0.001$	(a) - (b) $P < 0.02$ (a) - (c) $P < 0.001$		
4. No addition	(a) 2.49 ± 0.19	6.27 ± 0.52	3.78	< 0.001
L-Alanine (2 mM)	(b) 1.56 ± 0.11	2.65 ± 0.27	1.09	$= 0.01$
L-Alanine (20 mM)	(c) 0.95 ± 0.024	1.09 ± 0.051	0.14	$= 0.05$
Significance of differences	(a) - (b) $P < 0.01$ (a) - (c) $P < 0.001$	(a) - (b) $P < 0.001$ (a) - (c) $P < 0.001$		

tions in which protein synthesis is prevented, there can be no effect of insulin in increasing the entry of amino acids into protein. Therefore, according to the hypothesis, no effect of insulin on the accumulation of aminoisobutyric acid should be observable.

Of the various compounds available which might be expected to inhibit amino acid incorporation, general metabolic inhibitors (cyanide, azide, etc.) are not suitable for the present purpose, for although they effectively inhibit this process (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Manchester & Young, 1959) they also interfere with amino acid accumulation. Amino acid analogues might also be expected to interfere with amino acid accumulation and are therefore unsuitable potential inhibitors of protein synthesis (though neither DL-ethionine nor DL-norleucine at concentration of 20 mM has been found to decrease incorporation of [14 C]glycine). Unfortunately it has not proved possible to find any compound which will specifically inhibit amino acid incorporation without interfering with other metabolic activities and energy-producing reactions of the cell as judged by appearance, potassium release and glucose uptake (Randle & Smith, 1958). Neither chloramphenicol nor 8-azaguanine, which have been widely used as inhibitors of protein synthesis in bacterial systems, had any observable effect on amino acid incorporation into protein of diaphragm (Table 10). Since 8-azaguanine had no effect on incorporation of [14 C]adenine into nucleic acid either, it may be that it is unable to penetrate into

diaphragm to the site of nucleic acid synthesis. 5-Fluoro-6-deoxyuracil, which has been found to interfere with synthesis of deoxyribonucleic acid (Flaks & Cohen, 1959), was without effect on amino acid incorporation in the short duration of these experiments (Table 10). Manchester & Young (1958b) found that tolbutamide inhibited amino acid incorporation, but at the concentrations required to observe this effect tolbutamide also stimulated release of potassium and uptake of glucose (Table 10). 1:2-Dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzenes, which has been used by Woolley (1952) as a growth inhibitor, severely inhibited incorporation at 10 μ M concentration and above, but, as with tolbutamide, at this concentration it also stimulated release of potassium and uptake of glucose (Table 10). Proflavin has long been known as a growth inhibitor in bacterial systems. At 20 μ g./ml. it appeared to be inhibitory towards amino acid incorporation and to promote only a slight release of potassium. Since it is probable that the rate of amino acid incorporation in the presence of an inhibitor declines steadily throughout the first part of the incubation, in the subsequent experiments with proflavin (Table 11) diaphragm was first preincubated for 30 min. in buffer containing 20 μ g. of proflavin/ml. before being transferred to the final incubation medium containing [14 C]glycine or aminoiso[14 C]butyric acid, where the concentration of proflavin was 10 μ g./ml. Under these conditions there was a significant release of potassium

Table 10. *Effect of addition to the medium of various compounds, which can under certain circumstances inhibit protein synthesis, on incorporation of amino acid into protein, uptake of glucose, release of potassium and incorporation of nucleotide into nucleic acid of isolated rat diaphragm*

—, No observation was made. 'Stimulation' implies a significant effect. DCDNS, 1:2-Dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzene.

Additions to medium	Inhibition of incorporation of [14 C]glycine into protein (%)	Effect on uptake of glucose	Effect on release of potassium
Tolbutamide	83 (6)	Stimulation	Stimulation
Chloramphenicol (50 μ g./ml.)	7 (6)	—	—
(250 μ g./ml.)	18 (3)	—	—
8-Azaguanine (30 μ g./ml.)	0 (6)*	—	—
(300 μ g./ml.)	0 (3)*	—	—
DCDNS (100 μ M)	92 (6)†	No change	—
(10 μ M)	69 (6)	Stimulation	Stimulation
(1 μ M)	3 (6)	—	—
5-Fluoro-6-deoxyuracil (10 μ M)	7 (3)	—	—
Proflavin (100 μ g./ml.)	90 (3)*	—	—
(20 μ g./ml.)	62 (3)	—‡	Slight increase
(4 μ g./ml.)	29 (3)	—	No effect

* In these experiments 8-azaguanine was tested also for any effect on incorporation of [14 C]adenine into nucleic acid (see Methods). It had no effect at either concentration.

† Severe rigor.

‡ See Table 11.

Table 11. *Effect of proflavin in vitro* on the incorporation of [¹⁴C]glycine into protein, accumulation of α -aminoisobutyric acid, release of potassium and uptake of glucose by isolated rat diaphragm*

Each figure, except for release of potassium marked (†), is the mean $\pm$ s.e. of the mean of six observations.

	Glucose (2.5 mg./ ml.) added to the medium	Length of final incubation (hr.)	Additions to the medium				Significance of difference (<i>P</i>)
			Proflavin		Controls		
			No addition (a)	Insulin added (0.1 unit/ml.) (b)	No addition (c)	Insulin added (0.1 unit/ml.) (d)	
1. Incorporation of [¹⁴ C]glycine (counts/min./mg.)			4.31 ± 0.29		23.0 ± 0.82		(c) - (a) < 0.001
Release of potassium (μ-equiv./g. wet wt.)	+	2.0	11.0 ± 1.0		0.1 ± 1.0		(c) - (a) < 0.001
Uptake of glucose (mg./g. wet wt./hr.)			4.23 ± 0.21		2.46 ± 0.32		(a) - (c) = 0.001
2. Incorporation of [¹⁴ C]glycine (counts/min./mg.)	-	2.0	3.45 ± 0.21	5.20 ± 0.38	21.8 ± 1.17	38.4 ± 1.70	(b) - (a) < 0.01 (d) - (c) < 0.001 (c) - (a) < 0.001
Release of potassium (μ-equiv./g. wet wt.)†			23	20	16	12	
3. Accumulation of α-aminoisobutyric acid (concn. ratio)‡	-	1.5	2.23 ± 0.09	3.32 ± 0.24	2.65 ± 0.15	6.33 ± 0.35	(b) - (a) < 0.01 (d) - (c) < 0.001 (d) - (b) < 0.001 (c) - (a) < 0.05

Release of potassium (μ -equiv./g. wet wt.)†

24 11

* The hemidiaphragms were preincubated at 37° for 30 min. in a medium containing 20 μ g. of proflavin/ml., then transferred to the final medium which contained labelled materials $\pm$ insulin. Incubation in this medium was for the period stated in the Table.

† Sum of potassium released during preincubation and final incubation (single pooled observations).

† Ratio: $\frac{\text{Concn. of aminoisobutyric acid in tissue water}}{\text{Concn. of aminoisobutyric acid in incubation medium}}$.

and increase of glucose uptake (Table 11), but the release of potassium was not as large as was found with the nitrobenzene compound or tolbutamide. Incorporation of glycine into protein was decreased by over 80% (Table 11). Although a significant stimulation of accumulation of aminoisobutyric acid was still observable in the presence of proflavin, the size of the insulin effect was substantially less than that found with diaphragm without proflavin (Table 11). The amount of aminoisobutyric acid accumulation in the absence of insulin showed a small reduction in the presence of proflavin.

DISCUSSION

Effect of insulin on the rate of protein synthesis

Although insulin consistently and significantly depressed the release of amino acids by diaphragm during incubation (Tables 1 and 4), the size of the insulin effect, roughly a 10% decrease, is much smaller than the magnitude of the effect of insulin on incorporation of labelled amino acids into the protein of diaphragm (50–100%: Manchester & Young, 1958a; Wool & Krahl, 1959a) normally observed. There are several possible reasons for this. First, if a portion of the release results from damage to the tissue sustained during excision, this will tend to obscure the size of the insulin effect. Thus when the quantity of amino acids released in the first 15 min. is disregarded (Table 2), the size of the insulin effect rises to 18%. Ideally, the intact diaphragm preparation of Kipnis & Cori (1957), whose cell pool of amino acids declines much less during incubation (Table 6), would be the more suitable system in which to measure the rate of release of amino acids as a result of protein catabolism, but with this preparation the concentration of amino acids in the medium rises to a higher value than with hemidiaphragms (Table 6), presumably because of the release of amino acids by the severed intercostal muscle. From the figure for the increase in the amino nitrogen content of the system during incubation (100 $\mu\text{g.}$ of amino nitrogen/g. of tissue/1.5 hr.; Table 6) the excess of protein breakdown over protein synthesis in hemidiaphragms can be calculated as $100 \times 6.25/1.5 = 415 \mu\text{g.}$ of protein/g. of tissue/hr. Secondly, although the ninhydrin colour reaction is fairly specific for amino acids (Moore & Stein, 1954), ninhydrin does react with ammonia and with peptides such as carnosine, anserine and glutathione. Determination of residual ninhydrin-reacting material after evaporation to dryness at pH 10 indicated that only small quantities of ammonia are released by diaphragm. The presence of substantial quantities of di- and tri-peptides, however, whose movements it is assumed are not

affected by insulin, might well be expected to mask the extent of the diminution by insulin of the release of amino acids when expressed as a percentage.

That insulin suppresses the rise in the total quantity of amino acids in the tissue and the medium (Tables 3 and 4) suggests that the hormone does bring about an increase in the amount of protein synthesized. It could be argued that a diminution in the rate of appearance of amino acids arises from a diminished rate of breakdown of protein rather than an increased rate of its synthesis, and it is not possible to say from the results whether insulin does or does not affect the rate of protein breakdown in diaphragm. [Opinion is divided about whether the rate of protein breakdown is altered in diabetes. Forker & Chaikoff (1952) concluded that the rate of protein catabolism of serum proteins was not markedly changed in diabetic dogs. Green & Miller (1960) found the rate of protein breakdown by the perfused diabetic liver to be elevated.] However, since amino acids incorporated into protein are not subsequently released on further incubation (Manchester, 1961), increased incorporation cannot result from decreased breakdown. In view of the stimulation by insulin of the incorporation of labelled amino acids into protein it seems very probable that at least part of the suppression of the appearance of amino acids must result from an increase in the rate of protein synthesis. From the figures in Table 3 the increase in the rate of protein synthesis brought about by insulin can be calculated as

$$(785 - 693) \times 6.25 = 575 \mu\text{g.}$$

of protein/g. of tissue/2 hr. If insulin stimulates the rate of protein synthesis by 50–100% then this gives a figure for the rate in the absence of added insulin of 575–1150 $\mu\text{g.}$ of protein/g. of tissue/2 hr., which agrees well with a corresponding figure of 750 $\mu\text{g.}$ estimated by Manchester, Randle & Young (1959) from figures obtained from the rate of incorporation of [^{14}C]glycine into protein of diaphragm.

The results included in Tables 1–4 are in conformity with and provide an understanding of the observation that insulin can suppress the rise of the blood amino acid concentration in the eviscerated animal (Mirsky, 1938; Frame & Russell, 1946; Ingle, Prestrud & Nezamis, 1947). As with the eviscerated animal (Frame & Russell, 1946; Ingle *et al.* 1947) and with the incorporation of labelled amino acids into protein of diaphragm (Manchester & Young, 1958a; Wool & Krahl, 1959a), the addition of glucose is without effect on the amounts of amino acids in the tissue and medium (Tables 1–5). The concentration ratios contained in Table 5 support the findings of

Manchester & Young (1960), Toyoda & Bessman (1960) and Guroff & Udenfriend (1961) that under the conditions in which insulin stimulates the accumulation of α -aminoiso[^{14}C]butyric acid it has no effect on the accumulation of naturally occurring [^{14}C]amino acids. It is a curious circumstance, however, which is not at present explicable, that the concentration ratios obtained here are very much higher than those observed earlier with [^{14}C]amino acids, which were of the same order as those obtained with aminoisobutyric acid in the absence of insulin (Manchester & Young, 1960). As Guroff & Udenfriend (1960) have recently shown for tyrosine, a [^{14}C]amino acid does not seem to equilibrate with more than a fraction of its own species contained in the cell pool.

Mechanism by which insulin enhances uptake of aminoisobutyric acid

Although the observation that addition of other amino acids depresses the effect of insulin on the accumulation of α -aminoisobutyric acid (Table 9) is consistent with the hypothesis that its enhanced accumulation in the presence of the hormone results from the increased entry of other amino acids into protein, in so far as significant effects of insulin on the accumulation of aminoisobutyric acid can be detected even in the presence of large concentrations of other amino acids the results could equally well be regarded as evidence inconsistent with this explanation. In the absence of insulin, as well as in its presence, addition of other amino acids depresses the accumulation of aminoisobutyric acid, though this is marked only at the 20 mM concentration. It is not possible to say from these figures whether the latter results arise from displacement of one amino acid by another or from competition for the systems which transport amino acids into the cell. On the other hand, in support of the hypothesis advanced, there is a remarkable correlation between the extra aminoisobutyric acid entering diaphragm in the presence of insulin and the extra amino acids incorporated into protein (see above). The average of the ratios for the accumulation of low concentrations of aminoisobutyric acid in the absence of other added amino acids is (from Tables 7 and 9) 4.82 in the presence and 2.27 in the absence of insulin. The extra quantity of aminoisobutyric acid accumulated in the presence of insulin in 1.5 hr. of incubation is therefore $(4.82 - 2.27) \times 0.22 = 5.6 \mu\text{moles/ml. of tissue water} = 4.2 \mu\text{moles/g. of tissue}$. Thus after 1.5 hr. the extra uptake of aminoisobutyric acid in the presence of insulin is equivalent to about 420 $\mu\text{g. of amino acids/g. of tissue}$.

In the experiments with proflavin (Table 11) it is not possible to rule out completely the possibility that part of the reduction in the accumulation of

aminoisobutyric acid in the presence of insulin results from an interference by proflavin with the energy-producing operations of the cell. In fact the stimulation of release of potassium and uptake of glucose suggests that this does occur to some extent. Nevertheless, the potassium release seemed less severe and the inhibition of incorporation of glycine greater than with either 1:2-dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzene or tolbutamide, and the uptake of aminoisobutyric acid in the absence of insulin was only slightly reduced. Unless the mechanism by which these compounds interfere with protein synthesis is entirely due to an inhibition of energy-rich phosphate production, it is a curious coincidence that the point at which they begin to interfere with protein synthesis is at the same concentration as that at which they interfere with other metabolic activities. It is possible that protein synthesis in diaphragm muscle is too closely integrated with the overall metabolism of the cell for it to be possible to find anything which can specifically inhibit this process without producing a more general interference.

The results of Tables 7–11 thus do not provide unequivocal evidence in favour of the available hypothesis for the mechanism by which insulin stimulates accumulation of α -aminoisobutyric acid. The findings that the effect of insulin on aminoisobutyric acid accumulation is markedly decreased both when its own concentration is raised (Table 7) and in the presence of high concentrations of other amino acids (Table 9), and under conditions in which amino acid incorporation is severely inhibited (Table 11), are, however, precisely those to be expected on the basis of the suggested explanation. Manchester & Young (1960) found that under the conditions in which insulin stimulated the accumulation of aminoiso[^{14}C]butyric acid there did seem to be a smaller stimulation of accumulation of [^{14}C]glycine, though not of other [^{14}C]amino acids studied. Since the concentration of glycine in the cell pool is higher than for most other amino acids (unpublished observations), it is possible that glycine can under these circumstances act to some extent as though it were a non-utilizable amino acid. On the other hand, Guroff & Udenfriend (1961) found that insulin had no effect on the accumulation of α -methyltyrosine, an α -methyl amino acid like α -aminoisobutyric acid, an observation which is not readily explicable on any basis at the present time.

Mechanism by which insulin stimulates protein synthesis

The precise mechanism by which insulin *in vitro* stimulates the incorporation of labelled amino acids

into protein of isolated tissues is at present unknown. Despite the pronounced stimulation by insulin of accumulation of α -aminoisobutyric acid, the lack of observable enhancement of the accumulation of other naturally occurring amino acids (Manchester & Young, 1960; Toyoda & Bessman, 1960) and of α -methyltyrosine (Guroff & Udenfriend, 1961), and the stimulation by insulin of incorporation into protein of amino acids whose transfer into the cell has already taken place (Wool & Krahl, 1959*b*; Manchester & Krahl, 1959), argue against a direct effect of insulin on the transfer of amino acids from the medium into the cell pool. That an effect of insulin on incorporation of glycine is still detectable when the concentration of glycine is raised to as high a figure as 20 mM (Table 8) is an observation again consistent with this view, for under these conditions the figures obtained with α -aminoisobutyric acid suggest that the rate of entry of amino acid into the cell is too large to be the limiting step.

Recent observations seem to indicate, however, that the pool of amino acids in diaphragm from which amino acids are taken for protein synthesis is not necessarily the same as the total amino acid pool of the tissue. Kipnis, Reiss & Helmreich (1961) have found that the time-curve for the incorporation of radioactivity into diaphragm protein is not what would be predicted from the rate of rise of specific activity of amino acids of the cell pool. Guroff & Udenfriend (1960) found that labelled tyrosine added to the medium exchanged with only a portion of the tyrosine already present in the pool of the diaphragm tissue. Thus the negative results of experiments designed to show an effect of insulin on the accumulation of amino acids under the conditions in which it stimulates accumulation of α -aminoisobutyric acid (Manchester & Young, 1960; Toyoda & Bessman, 1960; Guroff & Udenfriend, 1961) do not necessarily preclude the possibility that insulin enhances the entry of amino acids into a much more limited pool. Although the increased rate of incorporation of labelled amino acids into protein seems to result from an increased rate of protein synthesis rather than merely a facilitation of the transfer of labelled amino acids from the medium to the pool from which amino acids required for protein synthesis are drawn, if the rate of protein synthesis were limited by the availability of amino acids in this pool, then an action of insulin to enhance their transfer there, either from the medium or from the other pool or pools, would bring about an increased rate of protein synthesis. Alternatively, as has been put forward elsewhere (Battaglia, Manchester & Randle, 1960; Tepperman & Tepperman, 1960), if insulin accelerates transport of glucose in muscle by restricting the access of

energy-rich phosphate to a process concerned with the regulation of glucose transport (Randle & Smith, 1957), it may at the same time direct this energy-rich phosphate to the processes involved in protein synthesis whose rates of action are limited by the availability of energy-rich phosphate at this locus. At the present time it has not been possible to provide confirmation for either hypothesis.

SUMMARY

1. Insulin *in vitro* diminishes the rate of release of amino acids by isolated rat diaphragm during incubation. It does not affect the ratio of the distribution of amino acids between the tissue and the medium.

2. After incubation for 2 hr. the sum of the quantity of non-protein ninhydrin-reacting material contained in the tissue and that in the medium is less in the presence of insulin than in its absence. This observation is interpreted to mean that the enhanced rate of amino acid incorporation in the presence of insulin does represent an increased rate of synthesis of protein.

3. The possibility that the stimulation by insulin of accumulation of α -aminoisobutyric acid by diaphragm is secondary to an increased rate of protein synthesis which draws amino acids in the medium into the tissue has been examined.

4. As the concentration of α -aminoisobutyric acid is raised the effect of insulin on its accumulation by diaphragm disappears. Stimulation by insulin of incorporation of [14 C]glycine into protein is still observable with high concentrations of glycine.

5. In the presence of large concentrations of other amino acids the effect of insulin on the accumulation of α -aminoisobutyric acid is markedly reduced; under conditions in which the rate of amino acid incorporation is inhibited by proflavin the effect of insulin on the accumulation of α -aminoisobutyric acid is again markedly reduced.

6. These results are consistent with, though they do not provide unequivocal support for, the hypothesis advanced for the means by which insulin stimulates accumulation of α -aminoisobutyric acid.

7. The possibility that insulin influences either the access of amino acids to the site of protein synthesis or the availability of energy-rich phosphate required for protein formation is discussed.

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Corticosteroid 21-Sulphates in Human Urine

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Pasqualini (1958, 1960) studied the distribution of the corticosteroids between the free, sulphate and glucuronide fractions in the urine of subjects after administration of adrenocorticotrophic hormone and established that an important fraction of corticosteroids was excreted in the urine as conjugates, contrary to the usual opinion (Neher, 1958; Peterson, Wyngaarden, Guerra, Brodie & Bunin, 1955; Peterson & Pierce, 1960). Corticosterone and 11-dehydrocorticosterone are found mainly in the ester sulphate fraction. The aim of the present research was to demonstrate the existence of corticosterone 21-sulphate and cortisol 21-sulphate in human urine.

EXPERIMENTAL AND RESULTS

Paper chromatography. Table 1 gives the *R* values of synthetic corticosteroid 21-sulphates relative to that of deoxycorticosterone 21-sulphate (Pasqualini, Zelnik & Jayle, 1960). For hydrolysis of the conjugates the developed dried chromatogram was suspended at 40° for 3.5 hr. in a covered cylindrical glass jar containing a mixture of conc. hydrochloric acid and dioxan (1:3, v/v) (Schneider & Lewbart, 1956).

Identification of corticosterone sulphate

Corticosterone was administered orally to a normal subject in three 100 mg. doses at 8 hr. intervals. The urine was collected during the 48 hr. after the first dose. The